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Motor Learning and Neuroplasticity in an Aged Mouse Model of Cerebral Ischemia

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Motor Learning and Neuroplasticity in an Aged Mouse Model of Cerebral Ischemia

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Dedication

To Jack, for being there through everything.

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Motor Learning and Neuroplasticity in an Aged Mouse Model of Cerebral Ischemia

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Stroke is the leading cause of long-lasting disability in the United States and disproportionately affects adults in later life. Age-related decreases in dexterity and neural plasticity may contribute to the poorer prognosis of older stroke survivors, even following rehabilitative physical therapy. The goal of these dissertation studies is to determine how the cortical plasticity underlying motor skill learning, both before and after brain injury, changes in the aged brain.

The general hypothesis of these studies is that age-related changes in motor performance and the limited ability to regain function following brain injury are associated with dysfunctional plasticity of the forelimb representation in the motor cortex. This hypothesis was tested in intact C57BL/6 mice by training them on a skilled reaching task and deriving intracortical microstimulation evoked motor cortical representations of the forelimb to determine training-induced changes in the function of

the motor cortex. After ischemic lesions, age-dependencies in the effects of rehabilitative training in skilled reaching on forelimb motor cortical representations were investigated. Prior to injury, intact young and aged mice learned a skilled reaching task in similar time frames and with similar success rates. Training-induced reorganization in the young mouse motor cortex occurred in the caudal forelimb area, which is homologous to the primary motor cortex of primates. However, the rostral forelimb area, a potential premotor cortex, was larger in aged mice compared to young mice. Following focal ischemic lesions of the forelimb area of the sensorimotor cortex, aged mice had larger lesions and were more impaired than young mice, but both groups regained reaching ability after 9 weeks of rehabilitative training. Post-operative training resulted in plasticity of the rostral forelimb area in young mice, but we failed to see reorganization in the forelimb map of aged mice following rehabilitative training.

These dissertation studies suggest that more severe brain damage in response to ischemia leads to poorer outcome in aged animals. Although the reorganization of motor cortex following initial skill learning and relearning following brain damage changes with age, the ability to learn motor tasks and improve function with rehabilitative training is maintained in healthy aging.

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Chapter 1: Introduction

Increasing age is associated with a myriad of health conditions and increased risk for disease states, such as stroke, Parkinson's Disease, Huntington's Disease, and Amyotrophic Lateral Sclerosis, which directly affect the functioning of the motor system. Research using animal models of aging is shedding light on the neurobiological processes that underlie decreased motor performance as a consequence of both normal aging and age-related disorders. Of particular interest are the abilities and limitations of the aged brain during recovery of function following unilateral stroke, as this a leading cause of long-term disability in older Americans (Roger et al., 2011). However, the large majority of neuroscience research on stroke-like injury is conducted in young animals rather than aged animals, though there is a difference in the way the aging brain responds to damage compared to young adult animals (Yager et al., 2006; Alaverdashvili and Whishaw, 2010; Soleman et al., 2010). Even in the absence of disease or disorder, the aged brain typically shows a gradual degradation of motor function (Krampe, 2002; Burke and Barnes, 2006; Seidler et al., 2010). The goal of these dissertation studies is to determine how the healthy aged brain learns new motor skills and if rehabilitative training after focal ischemic lesions is effective in aged animals. This research is based on the hypothesis that age-related changes in motor performance, both before and after brain injury, are associated with dysfunctional plasticity of the forelimb representation in the motor cortex.

In order to answer these questions, we developed an animal model of motor cortical ischemia that produced deficits in forelimb use in young and aged mice. This

enabled us to study the effects of rehabilitation on aged animals following focal ischemic lesions of the forelimb area of the sensorimotor cortex. We chose to study forelimb deficits because these tend to be common, chronic, and difficult to rehabilitate through physical therapy in human stroke survivors (for recent examples, see Brunner et al., 2011; Faria-Fortini et al., 2011; Lin and Yan, 2011; Page et al., 2011). C57BL/6 mice were chosen as the preferred model for these studies because of their highly dexterous use of the forepaws and ability to learn skilled reaching tasks (Farr and Whishaw, 2002), their extended health into old age (Turturro et al., 1999) and the availability of transgenic mice with C57BL/6 backgrounds, including those that express fluorescent proteins or channelrhodopsin-2 in layer V neurons of the motor cortex (Ayling et al., 2009; Xu et al., 2009), which makes establishing this model useful for future studies. We chose to refine the analysis of intracortical microstimulation (ICMS) evoked motor maps in mice to establish a quantitative measure of reorganization of motor cortical representations.

The first experiment described, in detail, the organization of the motor cortex of young mice through ICMS and cytoarchitecture. The purpose of this experiment was to more fully develop previous descriptions of the mouse motor cortex (Caviness, 1975; Li and Waters, 1991; Pronichev and Lenknov, 1998) to establish a quantitative characterization of the baseline organization of the young C57BL/6 mouse motor cortical representation. An additional goal of this experiment was to determine similarities and differences between the motor maps of rats and mice. This will allow us to relate our ICMS results in mice to the many studies that have investigated reorganization of the motor cortex in rats and monkeys. Additionally, because ICMS is related to transcranial

magnetic stimulation (TMS), we can relate our mouse motor cortical map changes to those of humans.

The second experiment assessed the effect of aging on motor learning induced plasticity of the motor cortex. This study was based on the hypothesis that the ability to learn and perform skilled reaching tasks lessens during old age because of dysfunction in the learning-induced reorganization of the forelimb motor representations. An additional goal of this study was to determine how long-term practice on a skilled reaching task affects map reorganization and if aging changes the way the motor cortex responds to long duration training.

The third experiment assessed the forelimb impairments caused by endothelin-1 (ET-1) induced lesions of the sensorimotor cortex in young mice. ET-1 has been successfully used to produce focal ischemic lesions of the sensorimotor cortex in young and middle-aged rats (Adkins et al., 2004; Maldonado et al., 2008; Kim and Jones, 2010). The purpose of this study was to establish a reproducible model of focal ischemia in C57BL/6 mice that results in sustained behavioral deficits on reach-to-grasp and other sensorimotor tasks.

The final experiment compared the behavioral responsiveness to rehabilitative training and resulting cortical map plasticity of young and aged animals following focal ischemic lesions of the SMC. Additionally, we evaluated the efficacy of different types of rehabilitation on promoting behavioral improvement and neural plasticity in young and aged animals. Two different types of reach training commonly used with rodent models of post-stroke motor rehabilitation (Ballermann et al., 2001; Metz et al., 2001; Teskey et

al., 2003; Gharbawie et al., 2005; Maldonado et al., 2008) were tested to determine if certain types of rehabilitative training are more efficacious in aged animals. This experiment was based on the hypothesis that aged animals are less able to regain function following brain injury, even when provided with rehabilitative training, due to dysfunction in the reorganization of forelimb motor representations in peri-lesion cortex.

The remainder of this chapter provides the background information that forms the basis of the current hypothesis. The first sections describe the anatomy of the motor cortex and the effects of motor skill learning on the intact brain. The next sections describe the behavioral and neural changes that occur in the motor system during aging. The final sections describe the effect of stroke on the brain, and how the aged brain reacts differently to stroke compared to the young brain.

1.1 Anatomy of the mouse sensorimotor cortex

Unlike the rat, there are few studies of the organization of the mouse sensorimotor cortex (SMC). In rats, motor movements can be evoked over a large area of frontal cortex. The forelimb motor cortical representation of the rat is comprised of a large caudal forelimb area (CFA), primarily located in the lateral agranular cortex (AGl), and a smaller rostral forelimb area (RFA) within the medial agranular cortex (AGm). These areas are typically separated by an area from which movements of the neck or vibrissa can be elicited. The CFA is homologous to the primate primary motor cortex (MI) and the RFA is a putative homolog to the primate premotor cortex. The majority of corticospinal neurons project from the AGl (Donoghue and Wise, 1982; Bates and

Killackey, 1984; Neafsey et al. 1986), although a smaller population of corticospinal projections is found within the AGm as well.

The hindlimb and a portion of the forelimb motor representations in the primary motor cortex (MI) overlap with their respective sensory representations in primary somatosensory cortex (SI), which is found in granular cortex (G) (Hall and Lindholm 1974; Donoghue and Wise 1982). This overlap zone (OL) is characterized cytoarchitecturally by the presence of both densely packed granule cells in layer IV and large, widely spaced layer V pyramidal cells. Although this overlap zone has yet to be fully characterized in the mouse, Caviness (1975) described an area in the caudal transition between fields 6 and 4 (described as the MI representation of the paws) that is marked by an increase in the prominence of layers III and IV, the granular cell layers characteristic of sensory cortex, and a narrowing of layer V, which contains corticospinal projecting pyramidal neurons. Also in this area, fields 4, 1 (SI representation of the trunk and proximal extremities) and 3 (barrel cortex) are striated. Cytoarchitecturally and functionally, this area seems to be a potential overlap zone in the mouse SMC, but a more detailed analysis of this area still needs to be done. Chapter 2 works towards this by describing the correspondence of this area with movement representations.

Caviness (1975) described the cytoarchitecture of the neocortex of the mouse in the most detail done to date. Although his description of motor areas in the frontal cortical fields is highly influenced by findings from intracortical microstimulation studies in the rat, the ICMS-evoked motor maps of mice conducted by Li and Waters (1991) are consistent with his distinctions between motor and somatosensory cortices in the brain.

Caviness' field 6, which is homologous in location and cytoarchitecture to the AGl of rats, spans across much of the dorsal rostral extent of the mouse cortex and corresponds to the AGl as delineated by Li and Waters (1991), who found that motor movements can be evoked by ICMS within this area. The rostal most portion of field 6 (homologous to the rostral most portion of the rat AGl) and the whole of field 8 (homologous to the rat AGm), located along the dorsal medial edge of the cortex, correspond to the RFA in rats (Neafsey and Sievert, 1982). Li and Waters (1991) were able to evoke forelimb movements within this area of cortex in some animals, providing evidence for the existence of a RFA in the mouse motor cortex.

In the rat, both the MI and SI components of the SMC receive afferent connections from the thalamus, which contain sensory and proprioceptive information from the body (Killackey, 1973; Donoghue and Parham, 1983; Killackey and Sherman, 2003). SI sends projections to the striatum (McGeorge and Faull, 1989; Donoghue and Parham, 1983), superior colliculus (Wise and Jones, 1977), pontine nuclei (Wiesendanger and Wiesendanger, 1982; Mihailoff et al., 1985) and MI (Wang and Kurata, 1998). MI sends the majority of its projections to the spinal cord (Leong, 1983; Bates and Killackey, 1984; Miller, 1987), but also projects to the pons (Legg et al., 1989), striatum (Cospito and Kultas-Ilinsky, 1981; Donoghue and Kitai, 1981), basiler pontine nucleus (Mihailoff et al., 1985), reticular formation (Valverde, 1966) and SI (Donoghue and Parham, 1983).

Interconnections between MI and SI mainly occur in layer II/III horizontal cortical connections (Jones et al., 1979). In primates and cats, SI neurons synapse onto layer II/III neurons of MI (Jones et al., 1979). In cats, layer II/III cells synapse onto layer

V neurons (Asanuma and Rosen, 1972), which then send projections to SI dysgranular areas (White and DeAmias, 1977; Chapin et al. 1987). Interestingly, the projections from MI to SI originate only from the CFA, not from the RFA (Sievert and Neafsey, 1986). Within the forelimb motor area, the CFA receives input from the RFA and the main afferent inputs to RFA originate from the CFA (Wang and Kurata, 1998).

The topographical organization of movement in motor cortex is resolved using ICMS, which elicits movements within MI by stimulating corticospinal connections both directly and transynaptically (Stoney et al., 1968; Jankowska et al., 1975). It is thought that the movements are evoked due to transynaptic activation of pyramidal cells near the area of stimulation because repeated stimulation of the same site decreases the movement threshold (Jankowska et al., 1975) and increases the size of movement representations (Nudo et al., 1990). Additionally, Young et al. (2011) have recently found that movements can be elicited from both rat and mouse motor cortices by stimulating throughout the depths of layers II to VI. This provides additional evidence that intracortical connections mediate changes in activity patterns and connectivity. Intracortical connections also appear to mediate motor skill learning, as described below.

1.2 Motor skill learning in the intact brain

Acquisition of a new motor skill requires the formation of novel movement sequences and increased dexterity of the body parts involved in the new movements (Hammond, 2002; Monfils et al., 2005). Skill learning results in a cascade of plastic events within the motor cortex. Initially, beginning as early as one hour after the first

training session on a new task, dendrites begin to add new spines. During continued training, pruning of newly formed spines occurs, and further practicing of the task results in stabilization of a population of these newly formed spines (Xu et al., 2009). Newly formed spines are likely indicative of an increase in synapse number because the majority of cortical spines are axospinous and most spines (in contrast to filopodia) have a synapse (Yu and Zuo, 2011). Corresponding evidence shows that after 7 days of reach training, new synapses are found within the motor cortex (Kleim et al., 2004), within the site of ICMS derived motor map changes (CFA), but not in areas that did not reorganize (RFA and hindlimb representations; Kleim et al., 2002a). In addition to spinogenesis, synaptogenesis and dendritic branching (Greenough et al., 1985; Withers and Greenough, 1989), many proteins and genes are upregulated during the first days of training, such as the immediate early gene Fos (Kleim et al., 1996) and the neurotrophic factor BDNF (Fritsch et al., 2010). The process underlying reorganization of the motor map may be partially mediated by an LTP-like mechanism (Monfils and Teskey, 2004). Layer II/III horizontal connections form an interconnected network between different regions of MI and relay excitatory information to layer V neurons (Aroniadou & Keller 1993). Training results in a long-term increase in field potentials and a resultant increase in the synaptic modification range that allows for additional LTP (Rioult-Pedotti et al., 2007). A short duration of reach training (3-5 days) increases field potentials recorded from layer II/III of the trained motor cortex (Rioult-Pedotti et al., 1998) and reduction in the amount of LTP that can be elicited by stimulation of the trained cortex (Rioult-Pedotti et al., 2000). These structural and functional changes are thought to lead to strengthening of the

connections between neurons within the motor networks of the brain, a proposed mechanism of engram formation in the motor cortex (Monfils et al., 2005).

While the processes of spinogenesis and gene and protein upregulation occur during the behavioral acquisition phase of motor learning, functional reorganization of the motor map, at least as detected with ICMS, occurs during the maintenance period, after the skill has been mastered (Kleim et al. 2004). The expansion of representations of trained movements is thought to reflect the additional dexterity necessary to perform the newly learned skill. However, this expansion is not necessary for future performance of the task. When training is stopped following acquisition of the task, the motor map returns to baseline organization, but the ability to perform the skill remains (Molina-Luna et al., 2008).

The body representation in the motor cortex is organized into overlapping areas corresponding to different body parts. Yet, within a given body part, such as the forelimb, the organization of specific portions (shoulder, elbow, wrist, and digit) are intermixed (Neafsey et al. 1986). The interconnected nature of the motor representation is thought to provide the flexibility necessary to modify the existing network to accommodate behavioral change (Sanes and Donoghue, 2000). However, stimuli must meet certain criteria in order to induce plasticity of the motor map. Reorganization of the motor representation occurs when a novel movement sequence is learned in order to perform a new motor task. Although less work has been done on the effect of motor skill training beyond the cortex, plasticity has been found in the spinal cord following training paradigms that have failed to result in reorganization of forelimb movement

representations, such as operant conditioning of motor behaviors, and strength or endurance training (for review see Adkins et al., 2006).

Under baseline conditions, movement representations of more dexterous body parts (i.e., the hands and digits in humans) tend to have larger areas in the motor cortex. Plasticity in the intracortical connections between different forelimb movement representation areas is thought to reflect the recruitment of neurons in the cortical territory devoted to producing novel movement sequences necessary for performing a newly learned motor skill (Keller, 1993). At least in the absence of ongoing practice, learning-induced changes in the motor cortex return to baseline levels, despite retention of the skill (Molina-Luna et al., 2008). Thus, the lasting reorganization of the cortex is not necessary for continued performance of a motor skill. However, stability of new spines formed during motor learning (Xu et al., 2009) is a long-term change that may support continued skill performance. The mechanisms underlying motor map plasticity and the conditions under which the motor map reorganizes are far from being fully understood. However, ICMS-induced motor maps are relevant for relating motor cortical changes in rodents and monkeys to human studies of motor map plasticity using transcranial magnetic stimulation (TMS; Kleim et al., 2007), which, like ICMS, produces movements by direct and transynaptic activation of corticospinal neurons (Epstein et al., 1990). One pertinent question that remains to be answered is the role of motor cortical reorganization in extended practice on a task. Many people who learn a motor skill to the point at which performance becomes asymptotic continue using the skill on a regular basis, such as musicians and athletes (Lotze et al., 2003; Ajemian et al., 2010). While it

has been shown that motor maps return to baseline following a period without motor training (Molina-Luna et al., 2008), it is unknown what changes in motor cortical reorganization occur when a skill is continually used.

1.3 Motor skill learning during aging

Fine motor performance of the hands and digits begins to worsen around age 60 during healthy aging in humans (Smith et al., 1999). Motor performance also varies with the level of task complexity, in that more highly skilled tasks are more difficult for older adults to complete (Light and Spirduso 1990: Smith et al., 1999; Moore et al., 2010). Adults over the age of 60 show less ability to control the amount of force and coordination of individual digits during a finger-pinch task (Keogh et al., 2006) and tend to use fewer alternative grasping patterns than young adults (Wong and Whishaw, 2004). Increasing age is also related to a decrease in hand dominance, resulting in a more balanced bilateral use of both hands (Kalisch et al., 2006). Decreasing dexterity of the hands and digits results in trouble performing tasks of daily living, leading to a loss of independence. It is thought that substantial loss of motor dexterity is an early predictor of cognitive decline in subjects with mild cognitive impairment (MCI) and Alzheimer's Disease (AD; Kluger et al., 1997). MCI and mild AD patients perform more poorly than healthy older adults on tests of fine or complex motor skills (i.e., pegboard, alternating hand movements), and AD patients additionally performed poorly on tests of gross motor function (i.e., movement speed, stability, and strength; Kluger et al., 1997). Thus, greater declines in motor function may be indicative of a more poorly aging brain.

At least in healthy subjects, the loss of dexterity associated with aging can be attenuated. Skilled training on a finger movement task improved the ability of older adults to control pinch force, hand steadiness, and manual speed, compared to untrained older adults (Ranganathan et al. 2001). Additionally, if older adults are taught strategies for motor learning, it becomes easier for them to learn new motor tasks (Seidler, 2007). However, the efficacy of training instructions is limited by the subject's level of innate motor control (Blumen et al., 2010), and instructions that are effective in younger adults are not as effective in older adults (de Bruin et al., 2009). Although motor skills training seems to help older adults attentuate age-related losses in motor ability, it is unknown to what degree motor cortical reorganization supports motor skill learning in the aged brain.

1.4 Motor cortical function of the aged brain

Age-related changes in motor function are associated with alterations in the function of the motor systems in the brain, including decreased activity of primary motor areas and less inter-hemispheric inhibition. Transcranial magnetic stimulation (TMS) and functional magnetic resonance imaging (fMRI) studies suggest that the aged brain compensates for decreased activity in primary motor regions by increasing activation elsewhere. Advancing age is associated with greater activation of a number of cortical and subcortical regions related to motor performance, particulary the contralateral premotor areas, ipsilateral primary motor cortex, and cerebellum (Tallelli et al., 2008; Ward and Frackowiak, 2003). In addition to motor areas, greater activation of sensory and cognitive areas (defined by increased hemodynamic responses) occurred during

motor task performance in older adults, and recruitment of all areas increases as a function of task complexity (Hutchinson et al., 2002). The increase in activation of cognitive and sensory areas during movement is thought to be indicative of a shift from automatic to controlled processing of movement, which may allow the brain to compensate for loss of function in primary motor cortex and produce motor behavior that is more similar to that seen in young adulthood (Heuninckx et al. 2005, 2008).

Similar to the process of healthy aging in humans, rodents undergo age-related changes in neural function. For example, as rats age, the somatosensory cortical representation goes through a gradual degradation in the size of glabrous receptive fields (of hairless skin, as on the ventral paw), which are replaced with non-glabrous receptive fields (of hairy skin, as on the dorsal paw; Coq and Xerri, 2000), and a slowing in response latencies of neurons within the forelimb and hindlimb maps. This degradation of the cutaneous somatosensory map coincides with impairments in walking behavior (David-Jürgens et al., 2008). However, when aged animals are housed in enriched environments from weaning to 23-28 months of age, the forelimb somatosensory map more closely resembles the maps of young rats housed in standard laboratory conditions (Coq and Xerri, 2001) and walking behavior is maintained at normal levels (David-Jürgens et al., 2008). In contrast to the somatosensory cortex, little is known about how the organization and plasticity of the rodent motor cortex changes with age and the influence of experience on these changes.

1.5 Stroke: behavioral and neural effects

There are two main types of stroke: ischemic, which occurs when there is an interruption in blood supply, and hemorrhagic, which occurs when there is breakage of a blood vessel and resultant bleeding in the brain. The large majority (85%) of strokes are ischemic (Roger et al., 2011) and the majority of survivable strokes are small (Carmichael, 2005). Typically, an ischemic lesion consists of a core, where there is complete cell death, and a surrounding penumbral zone in which cells may survive for some period, but are dysfunctional. Connected areas, such as contralateral homotopic cortex, may undergo dysfunction and remodeling in response to lost input from the lesion area (Reinecke et al., 1999; Hsu and Jones, 2006).

Ischemic damage to cerebral sensorimotor systems in humans, non-human primates, and rodents produce similar deficits, such as difficulty with movement, aim, grasping, walking, vocalizing and swallowing, depending on the area of the cortex that is damaged (Riley et al., 2011). Many stroke survivors experience sensorimotor impairments of the upper extremities. Deficits of the arms, hands, and digits tend to be chronic and difficult to rehabilitate through physical therapy in human stroke survivors (for recent examples, see Brunner et al., 2011; Faria-Fortini et al., 2011; Lin and Yan, 2011; Page et al., 2011). To compensate for motor impairments, humans and animals tend to rely more on the less-affected arm and hand. However, in rats, this hyperreliance has been shown to be detrimental to regaining function in the contralesional forelimb (Allred et al., 2005, Allred and Jones, 2008, Allred et al., 2010). Structured rehabilitation of the contralesional limb is currently one of the most effective ways to regain lost

function. Taub and colleagues have developed Constraint-Induced Movement Therapy (CIMT) to restrict the use of the ipsilesional limb by placing a mitt over that hand and forcing human stroke survivors to use their contralesional limb to perform rehabilitation tasks or activities of daily living (Mark and Taub, 2004; Wolf et al., 2008, 2010).

In rodents and non-human primates, ischemic lesions can be targeted to specific portions of the motor cortex, such as the functional representation of the forelimb. Animals that were previously trained on a skilled reaching task lose the ability to skillfully perform the task after lesions of the forelimb representation in the sensorimotor cortex (Nudo and Milliken, 1996; Whishaw, 2000; Adkins et al., 2004; Kleim et al., 2007). This impaired skill performance is homologous to human upper extremity impairment and has permitted the study of neural mechanisms of rehabilitative training effects in upper extremity function following ischemic lesions in non-human animals. Rehabilitative training involves recurrent practice (often daily) on a task that is designed to force the animal to use the contralesional or "impaired" limb to improve its function. The most commonly used forms of rehabilitative training in animal models involve daily training on a skilled reaching task, which may be a novel or previously acquired task. Generally, improvements in behavioral performance are measured throughout the training period, either through probe trials or other sensorimotor tasks.

Relearning lost behaviors through training of the contralesional forelimb instigates plasticity in the remaining perilesion cortex. Initially, following an ischemic lesion in the forelimb area of the sensorimotor cortex of monkeys, there is a loss of forelimb representation surrounding the lesion site. In the absence of rehabilitative

training, further loss of the forelimb motor map occurs and forelimb deficits persist (Nudo and Milliken, 1996). However, skilled reach training results in the maintenance of remaining movement representations and reorganization of movement representations into perilesion cortex, which is accompanied by improvement in forelimb function (Nudo et al., 1996b; Pineiro et al., 2001; Jaillard et al., 2005). Skill training seems to be especially useful for improving function. When the ipsilesional arm is restricted so that the animal is forced to use its contralesional limb, but no rehabilitative task is administered, the motor map does not show reorganization and there is a futher loss of forelimb representations, even in areas remote from the lesion, similar to effects in animals that receive no rehabilitation after the lesions (Friel et al., 2000). These findings indicate that increased daily limb use is not enough to induce motor map plasticity. Rather, structured skill training may be necessary for functional reorganization and behavioral improvement to occur. However, improvement on a behavioral task does not mean that the task is being performed in the same manner as it was prior to lesion induction. Animals typically develop compensatory strategies that allow them to perform tasks more successfully (when measured quantitatively) following brain injury, but movement abnormalities often persist following rehabilitative training, particularly in old age (Alaverdashvili and Whishaw, 2010: Moore et al., 2011).

1.6 Stroke and rehabilitation in the aged brain

Stroke is more prevalent and more detrimental in older adults, and people over the age of 70 tend to be more disabled and dependent before stroke onset (Pohjasvaara et al.,

1997; Kammersgaard et al., 2004; Rojas et al., 2007). Older age at stroke onset is associated with less gain in functional independence following rehabilitation, a longer duration between the diagnosis of stroke and the onset of physical therapy (Wang et al., 2011), and greater likelihood of being discharged to a nursing home or private caregiver (Falconer et al., 1994). Studies of older stroke survivors show that many are dissatisfied with the rehabilitation they received, citing that individual needs were not met and the transition from hospital to home was difficult (Sabari et al., 2000; Talbot et al., 2004; Vincent et al., 2007). Thus, more research is needed to determine effective therapies for use in elderly populations.

Aged animal models of stroke are important for determining the efficacy of rehabilitative therapies for promoting behavioral improvements and beneficial neural plasticity. Following experimental induction of stroke, aged rats show long-lasting performance deficits in coordinated limb use during walking, limb use for postural support, and sensorimotor forelimb use asymmetries (Soleman et al., 2010). The duration of behavioral impairment also increases with age at stroke onset (Brown et al, 2003).

Rehabilitative tasks, such as reach training, have been shown to be effective in promoting behavioral improvements in middle-aged and older rats and monkeys (Maldonado et al. 2008; Alaverdashvili and Whishaw, 2010: Moore et al., 2011). Both young and old animals develop compensatory movement strategies for reaching. However, while young animals' grasp patterns begin to normalize over time, older animals' do not (Alaverdashvili and Whishaw, 2010: Moore et al., 2011). Although post-operative training can be effective in improving the behavioral success of older animals

following ischemic lesions, external influences may exacerbate age-related functional deficits and affect behavioral improvement. Exposure to stressful situations (i.e., repeated restraint) during the rehabilitation period after ischemic lesions in aged rats caused their normally poorer performance to decline even further (Merrett et al., 2010). Thus, the particular circumstances in which rehabilitative training is optimized for aged individuals remains unclear.

1.7 Summary

The following dissertation studies seek to answer the question of how the healthy aged brain learns new motor skills and why relearning of motor skills after brain injury is more difficult in old age. This research is based on the hypothesis that age-related changes in motor performance and the limited ability to regain function following brain injury are associated with dysfunctional plasticity of the forelimb representation in the motor cortex. The background information presented in the above chapter provides insight on how healthy and stroke-affected aged brains differ from one another and from healthy and stroke-affected young brains.

These dissertation studies seek to answer the questions of how motor skill training in aged animals affects the reorganization of motor cortex typically associated with motor skill learning in young animals, what types of rehabilitative training are best able to support behavioral improvement in aged animals following focal ischemic lesions of the SMC, and how reorganization of lost motor representations differs between young and aged brains following rehabilitative training.

The first experiment in the following dissertation studies characterized the baseline organization of the young C57BL/6 mouse motor cortical representation. This study identified key differences between the motor maps of rats and mice and also served as a foundation for the remainder of these dissertation studies. The second experiment assessed the effect of aging and duration of training on motor learning induced plasticity of the motor cortex. This study resulted in the development of a model of motor learning in aged mice, which was the basis for the next two experiments. The third experiment assessed the forelimb impairments caused by ET-1 induced lesions of the sensorimotor cortex in young mice. This experiment allowed us to establish a reproducible model of focal ischemia in C57BL/6 mice and a sensitive set of behaviora assays for detecting motor impairments on which the final chapter was based. The final experiment compared the behavioral responsiveness to rehabilitative training and resulting cortical map plasticity of young and aged animals following focal ischemic lesions of the SMC. Additionally, we evaluated the efficacy of different types of rehabilitative training on promoting behavioral improvement and neural plasticity in young and aged animals.

The following dissertation studies provide a model system of healthy aging and stroke related to a common unavoidable risk factor: age. Hopefully, the results of these studies can help to shape future research and clinical rehabilitative practices with older stroke survivors. The aged brain does not necessary respond to stroke in the same way as the young brain, and it can not be expected that the same types of physical therapy that help younger (<60 year old) stroke survivors will help older (>60 year old) stroke survivors.

Chapter 2: The Organization of the Forelimb Representation of the C57BL/6 Mouse Motor Cortex as Defined by Intracortical Microstimulation and Cytoarchitecture

2.1 Abstract

The organization of forelimb representation areas of the monkey, cat, and rat motor cortices have been studied in depth, but its characterization in the mouse lags far behind. We used intracortical microstimulation (ICMS) and cytoarchitectonics to characterize the general organization of the C57BL/6 mouse motor cortex, and the forelimb representation in more detail. We found that the forelimb region spans a large area of frontal cortex, bordered primarily by vibrissa, neck, shoulder, and hindlimb representations. It included a large caudal forelimb area (CFA), dominated by digit representation, and a small rostral forelimb area (RFA), containing elbow and wrist representations. When the entire motor cortex was mapped, the forelimb was found to be the largest movement representation, followed by head and hindlimb representations. The ICMS defined motor cortex spanned cytoarchitecturally identified lateral agranular cortex (AGl) and also extended into medial agranular cortex (AGm). Forelimb and hindlimb representations extended into granular cortex (G) in a region that also had cytoarchitectural characteristics of AGl, consistent with the primary motor-somatosensory overlap zone characterized in rats. Thus, the mouse motor cortex has homologies with the rat in having two forelimb representations and an overlap zone, but is distinct in the predominance of digit representations.

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2.2 Introduction

The characterization of motor cortical organization in rats, cats and primates has facilitated a wealth of research on its role in movement and its reorganization and plasticity during motor skill acquisition and recovery from brain injury (reviewed in Monfils et al. 2005; Adkins et al. 2006; Nudo 2007). In contrast, there is a paucity of information on the organization of motor cortex in mice despite their increased use in studies of the neurobiological basis of behavior and disease states. Currently, the most widely used laboratory mouse brain atlas positions motor cortex based on extrapolations from rats (Paxinos and Franklin 2004), but the degree of homology between the motor maps of these two species has not been well established. Mice, like rats, use their forepaws in an extremely dexterous manner and this capacity is increasingly capitalized upon for studies of motor skill learning (e.g., Tucci et al. 2007; Xu et al. 2009) and models of upper extremity impairments after brain injury (Farr and Whishaw 2002; Menalled and Chesselet 2002; Carmichael 2005; Farr et al. 2006; Fleming and Chesselet 2006; Horie et al. 2008; Brown et al. 2009; Tennant and Jones 2009; Xiong et al. 2010). Thus, a better characterization of the organization of the forelimb representation of the motor cortex would be particularly useful for studies of motor cortical plasticity in mice.

The only well characterized rodent motor cortex is that of the rat. Rat cortex is separated into distinct subregions that can be discerned based on the organization of the

underlying cytoarchitecture and electrophysiological characteristics (Donoghue and Wise 1982). In the rat primary motor cortex (MI), cytoarchitectonics and electrophysiological approaches have been combined to clearly establish a relationship between cortical structural and functional organization. Using intracortical microstimulation mapping (ICMS), movements can be evoked in anesthetized animals via stimulation with a microelectrode placed in layer V of the motor cortex. Motor movements can be evoked over a large area of frontal cortex, specifically in the lateral agranular cortex (AGl), which contains corticospinal projecting neurons (Donoghue and Wise, 1982; Bates and Killackey, 1984; Neafsey et al. 1986). Fewer movements can be elicited from stimulation of the medial agranular cortex (AGm), although this area also has corticospinal projections. The forelimb area of the rat motor cortex is organized such that there is a large caudal forelimb area (CFA) and a smaller rostral forelimb area (RFA), each with corticospinal projecting neurons (Neafsey and Sievert 1982; Liang et al. 1993). The hindlimb and a portion of the forelimb motor representations overlap with their respective sensory representations in primary somatosensory cortex (SI), which is found in granular cortex (G) (Hall and Lindholm 1974; Donoghue and Wise 1982). This overlap zone (OL) is characterized cytoarchitecturally by the presence of both densely packed granule cells in layer IV and large, widely spaced layer V pyramidal cells.

Only two studies have been published describing the organization of the mouse motor cortex based on ICMS. The more detailed of these studies, by Li and Waters (1991), relates the ICMS generated maps to cytoarchitectonics, which previously had been described by Caviness (1975; see also Frost and Caviness 1980). However, Li and

Waters' study used a dystrophic mouse strain (dy2j/dy2j C57BL mice) that is characterized by muscle and peripheral nerve degeneration (Sunada et al. 1995), and it is unknown if it accurately represents the map of the wildtype mouse. They found that movements of the elbow, wrist, and digit can be elicited in an approximately 4 mm^2 cortical territory mostly anterior to bregma. The motor map was largely contained within the AGl, but the more caudal forelimb territory also extended laterally into part of the granular cortex (which contains SI), raising the possibility that, like rats, there may be a partial overlap between SI and MI in the forelimb representation. Pronichev and Lenkov (1998) conducted ICMS in mongrel white mice. They were able to elicit movements of the forelimb in an approximately 3 mm^2 area, but their maps extended less rostrally and more medially than that found by Li and Waters. They found no evidence of two motor maps for the forelimb. It is difficult to know whether to attribute the differences in these two studies to the mouse strains because the studies also used different anesthetic drugs (ketamine versus thiopental) and produced maps with different spatial resolutions (250 µm versus 500 µm stimulation distances; Li and Waters 1991 versus Pronichev and Lenkov 1998, respectively). A new minimally invasive motor mapping technique has recently been developed by Ayling et al. (2009) and Hira et al. (2009), but has not yet been used to describe the organization of the mouse motor cortex in detail, e.g., the internal organization of the forelimb area and its position relative to other movement representations.

Our purpose was to extend these previous characterizations of the mouse motor cortex to the wildtype C57BL/6 mice, one of the most commonly used mouse strains for

behavioral analyses and a popular background strain for the generation of transgenic animals. Furthermore, we sought to determine the internal organization of the forelimb area of the mouse motor cortex as well as the relationship of ICMS-induced movement representations to underlying cytoarchitecture. For the purpose of comparison with rat motor cortex (Monfils et al. 2005), we adapted the ICMS procedures used in rats (Adkins et al. 2006; Kleim et al. 1998, 2002, 2003, 2004) to generate high resolution motor maps in the mice. We used stimulation-evoked movements to characterize motor cortical organization to be consistent with the approach used for over a century to describe the motor map in humans, nonhuman primates, cats and rats (e.g., Roaf and Sherrington 1906; Brown and Sherrington 1911; Stoney et al. 1968; Nudo et al. 1996; Kleim et al. 1998; Eisner-Janowicz et al. 2008; Stepniewska et al. 2009).

2.3 Materials and methods

2.3.1 Subjects

Well-handled 3-5 month old male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were used. Mice were housed in groups of three to four in Polycarbonate cages with shavings and wire mesh top and were kept on a 12:12 h light/dark cycle. They received common standardized housing supplementation (a piece of PVC pipe as a cage enclosure, a cardboard roll for nesting, and small wooden beads and spools to chew). Similar cage supplementation has been found to reduce stereotypic and anxiety-related behaviors (Smith and Corrow 2005; Olsson and Sherwin 2006) and not to increase experimental variability or reproducibility (Wolfer et al. 2004). Nine mice were naïve to

any experimental manipulations. To enhance the generalizability of the results, an additional 18 mice that were used in other studies were also included (Tennant et al. 2009, 2010). These animals received various behavioral experiences which were found to not significantly influence ICMS results, as described below. The inclusion of animals with behavioral variability is intended to broaden the reproducibility of the anatomical characterization (Richter et al. 2010). This included mice (n=6) receiving 17-21 days of training on the Pasta Matrix Reaching Test (retrieving approximately 10-12 pieces/day). For the same time period, other mice received capellini pasta pieces (n=7) or bits of pasta too small to handle (n=5), but no reach training. During the reaching test period, mice were maintained on scheduled feeding (~3 g/day). A subset (n=6) were tested once weekly on the Ladder Rung and Bilateral Tactile Stimulation Tests, inclusive of those receiving reach training or pasta exposure. Note that the lack of a training effect on the maps in the current study *does not* indicate that these behavioral manipulations have no effect on the motor map of mice. It is well established that behavioral training can change movement representations in monkeys and rats (Nudo and Milliken 1996; Nudo et al. 1996; Kleim et al. 1998). While we did not see an effect of 17-21 days of behavioral training on the motor map, map changes are slow to occur and require repetitive practice to instigate (Kleim et al. 2004; Adkins et al. 2006) and recent data indicates that a substantially longer duration of training on these tests results in significant changes in movement representations (Tennant et al. 2010). Animal use was in accordance with a protocol approved by the University of Texas at Austin Animal Care and Use Committee.

2.3.2 Intracortical microstimulation (ICMS) mapping

Motor cortical representation areas were defined by the movements generated at the lowest stimulation thresholds, the approach traditionally used to characterize motor cortical maps (e.g., Nudo et al. 1996; Kleim et al. 1998). Animals were anesthetized with an initial cocktail of ketamine (150 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) that was supplemented with additional ketamine to maintain the plane of anesthesia. When necessary, isofluorane (0.5-1 % in oxygen) was used to bridge between ketamine injections, but no ICMS was conducted during isoflurane exposure (due to the loss of muscle tone caused by isofluorane). Each mouse was placed into a mouse stereotaxic frame (Stoelting, Wood Dale, IL). Lidocaine (2 mg/kg, s.c.) was injected into the scalp, and a midline incision was made. The cisterna magna was punctured to reduce cerebrospinal fluid volume (which thereby reduces cortical upwelling), and the skull and dura overlying the motor cortex were removed. The craniotomy was then filled with warm (37° C) silicone oil.

Using a computer interfaced digital stereomicroscope camera, a picture of the cortical surface was taken and overlaid, using Canvas software (ACD Systems International Inc.) with a grid with 250 µm by 250 µm intersection spacings. Sites of stimulation were at grid intersections, with the exception of minor adjustments in placement made to avoid penetrating surface vasculature. The mean number of stimulation sites for forelimb area-only maps was 63 ± 3 sites. For full MI maps, the mean was 136 ± 8 sites. The point of bregma was marked on the maps and this and other skull landmarks were used to aid alignment of grid coordinates with histological sections,

as described below. One grid axis was aligned approximately parallel with midline, which facilitated characterization of the spatial distribution of forelimb responsive sites across animals. Using a hydraulic micropositioner, intracortical penetrations with a glass microelectrode (20-25 μm tip diameter) with a platinum wire were made at a depth of 790-800 μm (corresponding to mid-to-deep layer V). Penetrations were made at each grid intersection until the entire extent of the forelimb representation was resolved, including the caudal and rostral aspects. At each site, a 40 ms train of 13, 200 μs, monophasic cathodal pulses was delivered at 350 Hz from an electrically isolated, constant current stimulator (BAK Electronics, Mount Airy, MD) at a rate of 1 Hz. Stimulation was increased up to a maximum of 60 μA, or until a visible movement was evoked on the contralateral side of the body. If a movement was evoked at or below 60 μA, the threshold current was determined by gradually decreasing the stimulation until the movement stopped. The lowest amount of stimulation required to evoke movement was considered to be the threshold current. If no movement was seen at 60 μA, the site was considered non-responsive. In cases where stimulation evoked more than one movement, the site was considered responsive to the movement that was determined to have the lowest threshold. For example, digit movements sometimes co-occurred with wrist movements at currents $\leq$60 μA, but if the wrist movement ceased and digit movements remained at reduced stimulation currents, then the site was recorded as a digit representation. Throughout the ICMS procedure, the forelimb was lightly supported between the elbow and shoulder, so that the paw was suspended freely, as used in prior rat ICMS studies (Kleim et al. 1998, 2002, 2004). This position was held constant across

animals. Movements were called by an experimenter that was blinded to the cortical position of the electrode. Movements were classified using methods that are well established in rat ICMS (Kleim et al., 1998, 2002, 2003, 2004). This included stimulation-timed localized movement at joints of the digit(s), wrist, elbow, shoulder, hindlimb or jaw, movement of any single or set of vibrissa(e), of torso (trunk) or tail, or of neck musculature (which was partially exposed by the incision). Based on previous experience with rat ICMS, we found the classification was quite applicable to stimulation-evoked movements observed in mice, with the possible exception of vibrissa and jaw movements (discussed below). Furthermore, it was feasible to apply in a sensitive and reproducible manner to the mice. The three experimenters recording movements (D.L.A., N.A.D. and A.L.A.) had high inter-rater reliability; there was no significant difference between experimenters in the number of sites (Fs=0.117-0.399, ps=0.890-0.675) or thresholds (Fs=0.012-0.417, ps=0.988-0.663) of each movement type per map.

Electrode penetrations were made in a systematic order to minimize the contribution of the mapping procedure to inter-animal variability. The first electrode penetration was made in the likely caudal forelimb area (CFA), and penetrations were made in 250 μm increments moving in an anterior direction until a non-responsive or non-forelimb movement was elicited. Forelimb movements were defined as movements at the elbow, wrist or digit joints. Penetrations were then continued medially from the last forelimb responsive site until a non-responsive or non-forelimb movement was elicited. Electrode penetrations were then made in the posterior direction, bordering in every

forelimb site with a non-responsive or non-forelimb site. This strategy was continued around the posterior, and then lateral, edges of the CFA. Finally, the interior of the map was filled in with penetrations every 250 μm. Borders were established first to minimize the tendency of stimulation to cause an expansion of the CFA (Nudo et al. 1990; Teskey et al. 2002). When the caudal forelimb area was completely mapped, electrode penetrations were made in an anterior direction until a forelimb movement was evoked in the rostral forelimb area (RFA). This area was then bordered and filled in as necessary.

In six animals, the entire MI was mapped, beginning with the CFA, then progressing to the RFA, vibrissa, neck/jaw, shoulder, trunk, hindlimb, and tail areas. All attempts were made to map each area as a distinct region or "mini-map" such that the same systematic method used in the CFA and RFA was used to first border and then fill in the remainder of the body representation. Shoulder is often considered to be part of the proximal forelimb representation (e.g., Neafsey et al. 1986; Kleim et al. 1998; Kleim et al. 2004) and shoulder movements contribute to forelimb movements, but also contribute to other body movements. For the purpose of characterizing forelimb movement representations specifically, we omitted the shoulder from the analyses focused on the forelimb representation, but consider the forelimb area with and without shoulder representation it in the analyses of the entire MI. Neck, jaw and vibrissa representations are considered together as a head movement representation in the analyses. This was done because the detection of vibrissa and jaw representations was variable between mice and it seems likely that their reliable detection requires further refinement of the rat-adapted ICMS methods. In the rat, the representations of neck and vibrissa overlap and

revealing vibrissa movements requires a more superficial anesthetic plane, evident by spontaneous whisking (Tandon et al., 2008). In the mice of this study, spontaneous whisking was frequently seen in the plane of anesthesia considered suitable for ICMS, but the onset of spontaneous tail flicking was a more consistent characteristic of emergence from anesthesia. Further study is needed to determine the mouse-specific ICMS conditions optimal for revealing the vibrissa representation. Substantial jaw representations are revealed in the rat by visible stimulus timed movements of the jaw (Neafsey et al. 1986; Kleim et al. 1998; Tandon et al. 2008). However, using this criterion, only 2 jaw movements were discernable in the first 5 mice receiving full MI maps. To investigate the possibility that jaw movements occurred but were not detectable, in the sixth map, we adhered cat whiskers to the lower jaw of the mouse to make the slightest movements more obvious. In anecdotal support for the possibility that jaw movements were occurring in the previous maps but too subtle to discern, a large jaw representation was revealed in this mouse (see Results).

Areal extents of movement representations were calculated by multiplying the number of points corresponding to a specific movement (e.g., elbow) by the area of a single grid square (0.0625 mm^2). To characterize the spatial distribution of the forelimb representation area across animals, a two dimensional frequency distribution of forelimb responsive sites at coordinates relative to bregma was also generated. Images of the skull were used to align maps relative to midline and bregma.

2.3.3 Histology

At the end of the ICMS procedure, animals were given an overdose of sodium pentobarbital (175 mg/kg, i.p.) and perfused intracardially with 0.1 M phosphate buffer and 4 % paraformaldehyde. The brains were allowed to post-fix for approximately 1 week before being sliced on a vibratome at a thickness of 50 μm. Every sixth section was mounted on a gelatin-subbed slide and Nissl stained with toluidine blue. An additional series from each brain was stained for myelin using Eriochrome Cyanine R (Kiernan 1984; Tester and Howland 2008). These sections were used to verify the depth of electrode penetrations and to delineate cortical subregions based on cytoarchitectonics.

2.3.4 Cytoarchitecture

A subset of animals (n=13) was used for cytoarchitectural analysis, inclusive of four animals in which the entire MI was mapped. Digital images of Nissl and myelin stained coronal sections were taken at a magnification of X51 using an Olympus BX61 microscope. The mouse cortex has cytoarchitectural characteristics similar to the rat sensorimotor cortex (Caviness 1975) and these characteristics were used to define boundaries of the medial agranular cortex (AGm), lateral agranular cortex (AGl), overlap zone (OL), and granular cortex (G) (Fig. 2.1).

The AGm is characterized by a compact layer II and a pale-staining layer III, the AGl is characterized by more homogenous superficial layers and a broad layer V that contains particularly large pyramidal cells, and G is characterized by the presence of densely packed granule cells in layer IV (Donoghue and Wise, 1982; Bates and

Killackey, 1984; Neafsey et al. 1986). The OL is characterized cytoarchitecturally by the presence of both densely packed granule cells in layer IV and large, widely spaced layer V pyramidal cells. In myelin stained sections, OL and G also have more darkly stained laminae than the adjacent AGl and AGm (Brecht et al. 2004).

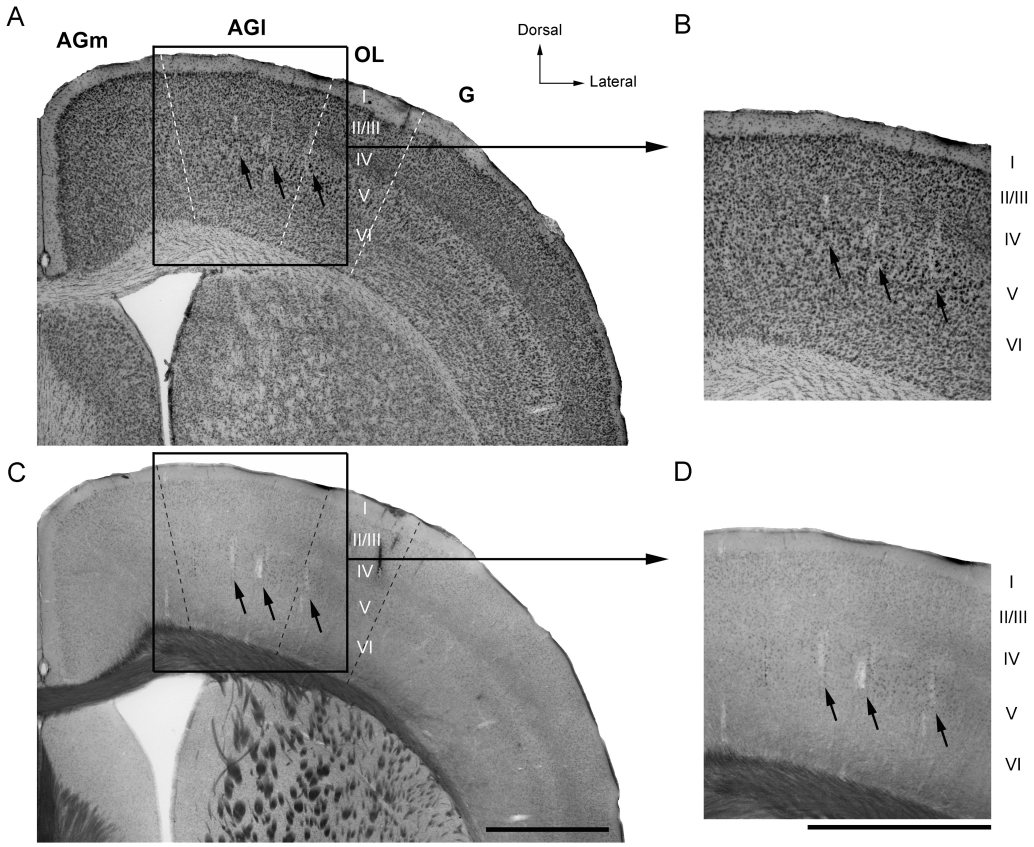


Figure 2.1 Representative coronal sections used to relate cytoarchitecture to ICMS generated maps. (A) Nissl stained section. Cytoarchitectural zones are separated with white dashed lines and layers are indicated with Roman numerals. Arrows point to electrode tracks ending in layer V. AGm, medial agranular cortex, AGl, lateral agranular cortex, OL, motor-somatosensory overlap zone, G, granular cortex. (B) Higher magnification of inset shown in A. (C) Myelin stained section. Cytoarchitectural zones are separated with black dashed lines. Electrode tracks parallel those in A (because of the use of fixed spacings between stimulation sites). (D) Higher magnification of inset shown in C. Scale bars equal 1 mm.

After matching for magnification, the ICMS generated maps were superimposed (as colored circles representing movements) onto the cytoarchitecturally delineated coronal section images using Canvas software (ACD Systems International Inc.). Using macrostructural landmarks and the coronal section coordinates reported in Paxinos and Franklin (2004), the anterior-posterior position of the coronal plane was determined and matched with the closest anterior-posterior map coordinate. Within each coronal section, colored circles corresponding to stimulation sites in the medial-lateral direction were then superimposed over the coronal section image to extend in layer V perpendicular to midline at 250 µm increments, adjusting depth as needed relative to the cortical surface. The closeness of alignment with coordinates using this method was verified in a subset of animals (n=7) in which a small injection of 1,1',di-octadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular probes, Inc., Eugene, OR), a lipophilic dye, was made just lateral to midline at the coronal plane of bregma, at the end of the ICMS procedure, before perfusion. An analysis of 300 visible electrode tracks in a subset of animals (n = 7, all naïve), confirmed the alignment of the coordinate system with cytoarchitecture and in the remaining animals the alignment was further verified by electrode tracks when they were evident in the section. For each movement, the number of responsive sites per cytoarchitectural region (AGm, AGl, OL or G) was counted for each animal, and a percentage of the movement representation falling into each region was calculated by dividing the number of sites per region by the total number of responsive sites.

2.3.5 Statistics

All statistical analyses were conducted using SPSS software. One-way analyses of variance (ANOVAs) were conducted to compare area, percentage of map, and movement thresholds across movement representations, with movement type as the independent variable and area, percentage of map, and threshold each as a separate dependent variable. Bonferroni post-hoc tests were conducted to further analyze group differences.

There were no significant differences in the forelimb representation areas of naïve mice and mice from previous studies that had received experience in behavioral tasks (see Subjects). The total areal extent of the rostral (RFA) and caudal forelimb (CFA) representations were similar between mice that were not exposed to behavioral tasks (naïve and untrained controls; CFA = 1.82 ± 0.12 mm^2; RFA = 0.20 ± 0.02 mm^2) and mice receiving practice in pasta handling or reaching (CFA = 1.74 ± 0.14 mm^2; RFA = 0.20 ± 0.03 mm^2). There were also no significant differences in the areal extents of movement subtypes (elbow, wrist, digit) between naïve/untrained and behaviorally experienced mice (*F*s = 0.058-1.25, *p*s = 0.81-0.27). The largest F value (*F*=1.25) was found for the digit representation when naïve/untrained mice were compared with the more experienced mice. The trained mice tended to have a smaller digit representation area than the other mice. However, power analyses indicate that 186 animals would be needed detect a significant difference ($p<0.05$) in digit representation area with 80% power. Furthermore, when these animals were omitted from the analyses, there was little impact on mean values or the pattern of inferential results. Thus for the purpose of capturing variance in the motor maps (Richter et al., 2010) and given the lack of any

significant difference between the groups in movement representation areas, all animals were included in the following analyses. As noted above, a longer duration of training has been found to result in significant effects on the motor maps (Tennant et al. 2010).

2.4 Results

2.4.1 General organization of motor cortex

In animals in which the entire motor cortex was mapped (n = 6), the map was organized in the anterior-posterior dimension with a large, centralized caudal forelimb area (CFA) that was rostrally bordered by neck/jaw and vibrissa and caudally by shoulder, trunk, hindlimb, and tail (Figs. 2.2, 2.3). The forelimb area, including elbow, wrist, and digit representations, was found to occupy a large portion of the motor map (Fig. 2.2A-B). Overall, 39 ± 3 % of the total motor map was comprised of forelimb representation. Of the other representations, the representations of the head (neck, jaw and vibrissa) and hindlimb movements were the most substantial, comprising 28 ± 4 % and 15 ± 5 % of the total map, respectively. The representations of the head consistently encompassed a large expanse of the anterior MI. Note that the ICMS methods used are likely to underestimate vibrissa and jaw representations and overestimate neck representations (see Methods). The vibrissa representation was detected in 16 of the 27 maps of the study, inclusive of 2 of the 6 full MI maps. When detected, it was in the same territory in which neck movements were found in the other mice (Figs. 2.2E-F, 2.3, 2.4), consistent with the overlap of vibrissa and neck representations reported in rats (Tandon et al. 2008). It extended along the medial edge of the motor map, and also wrapped

around the anterior and anterolateral aspects (Figs. 2.2E-F). This characteristic of the vibrissa representation was also evident in the borders of forelimb representation-only maps (Figs. 2.3, 2.4), described next. With the exception of the vibrissa representation, ipsilateral movement responses were never seen at the movement thresholds eliciting

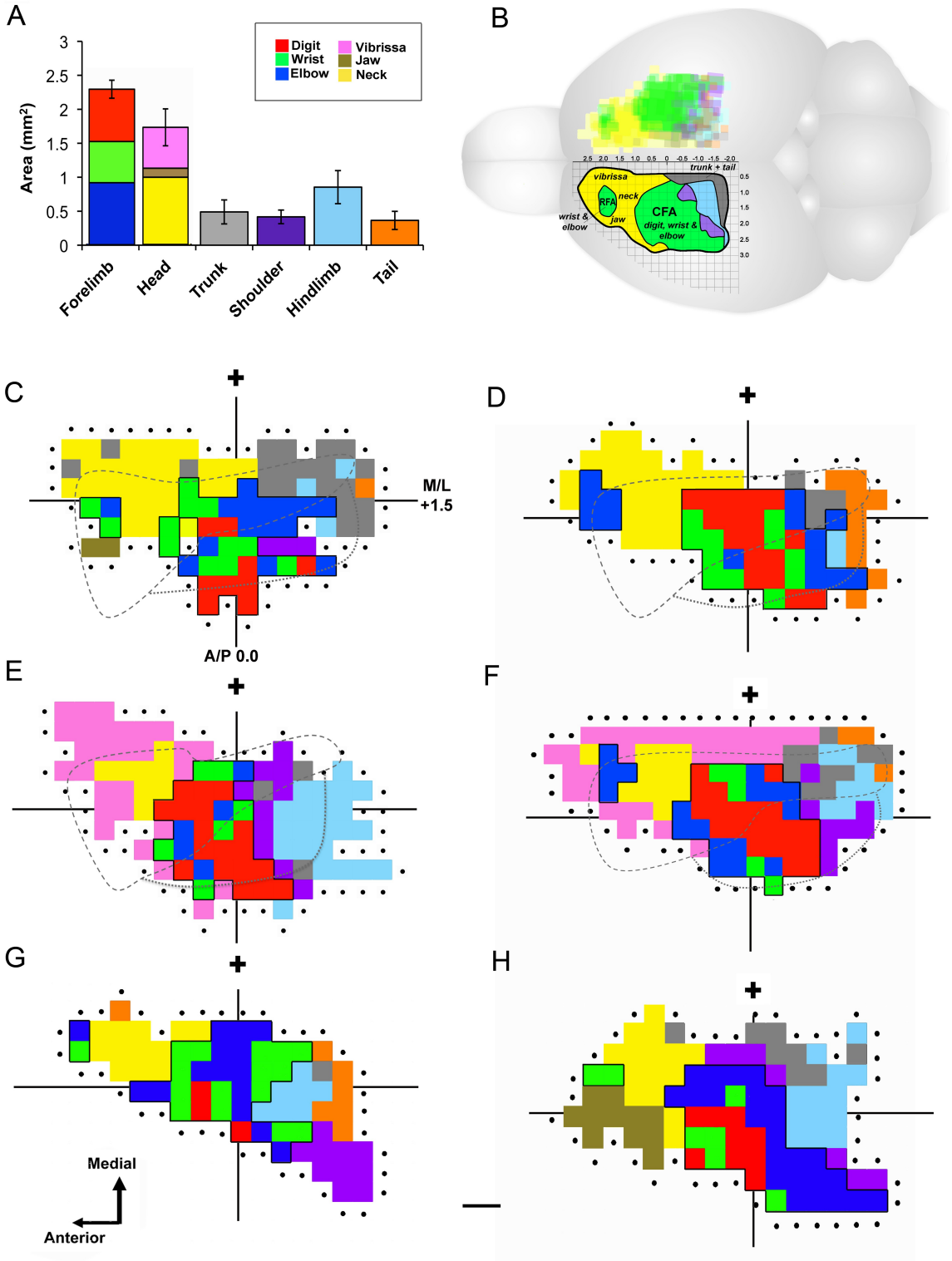

A
Digit
Wrist
Elbow
Vibrissa
Jaw
Neck
Area (mm²)
3
2.5
2
1.5
1
0.5
0
Forelimb
Head
Trunk
Shoulder
Hindlimb
Tail
B
vibrissa
neck
RFA
jaw
wrist & elbow
CFA
digit, wrist & elbow
trunk + tail
C
M/L +1.5
A/P 0.0
D
E
F
G
Medial
Anterior
H

Figure 2.2. Organization of mouse primary motor cortex (MI). (*A)* The areas of individual movement representations in mice with full MI maps (n=6). The forelimb representation was the largest of the movement representations. The error bars in the stacked columns represent the SEM of the total representation (summed across movement subtypes). *(B)* A schematic representation of the dorsal surface of the mouse cortex showing movement representation regions simplified by color coding all forelimb responsive sites as green and head movement representations as yellow. Transparent overlays of all maps in *C-H* are shown in the right hemisphere (top). The left hemisphere approximates their relative positions and sizes. Numbers are mm distances relative to bregma (point 0 on the horizontal axis). *(C-H)* All full MI motor maps. Colored squares represent movements evoked with a stimulating electrode placed into the center of the square. Map color codes for movement type in *C-H* match those of the bar graphs in *A*. Black dots are nonresponsive sites. *C-F* are from brains included in the cytoarchitectonic analyses. In all maps, large expanses of head representations were found in the anterior extent of the map, interrupted by an island of RFA, with the exception of the map in *E*, which lacked a discernable RFA. In *E* and *F*, large vibrissae representations were revealed in areas in which neck movements were found in other mice. In *H,* A substantial jaw representation was revealed in a mouse with cat whiskers adhered to the chin to make jaw movements more obvious. See text for details on the variability in the areal extent of the vibrissa and jaw representation. In all maps, "+" represents bregma (A/P 0.0) and the horizontal line extending below the map is 1.5 mm lateral to midline (M/L + 1.5). Long dashed outlines indicate the borders of AGl and short dashed outlines indicate the borders of OL. AGm is medial and anterior to AGl and G is lateral and posterior to OL. Scale bar equals 500 μm (the distance across 2 stimulation points).

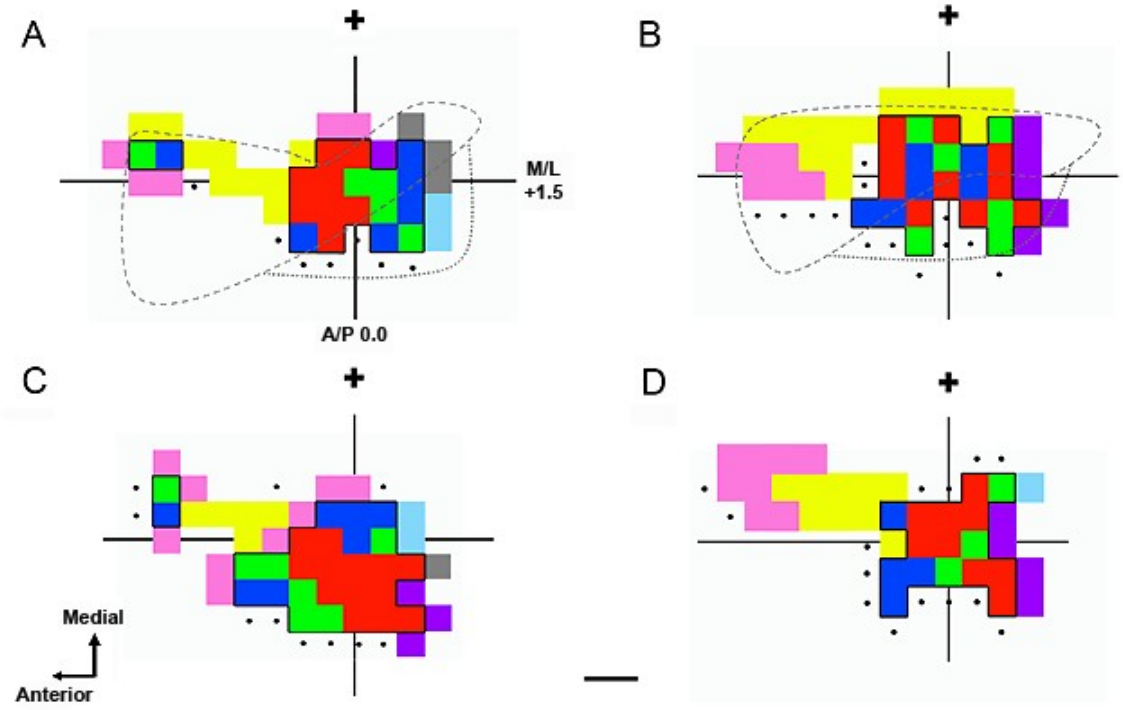


Figure 2.3 Additional examples of forelimb area maps. Dotted lines in A-B indicate cytoarchitectural boundaries overlaid on the motor map as in Figure 2.2. RFA was discernable in A and C, but not in B and D. Maps are typical in having large digit representations (red) in CFA and, when detected, a small RFA composed of wrist (green) and elbow (blue) representations surrounded by representations of the head (yellow, neck; pink, vibrissa). Color codes for movement representations are the same as in Figs. 2.2, 2.4. In anterior/posterior (A/P) and lateral (L) coordinates in mm relative to bregma (+), forelimb representations are shown between approximately (A) +2.2 to -0.7 A/P and 1-2 L, (B) +1 to -1 A/P and 0.75-2.2 L, (C) +2 to -0.7 A/P and 0.75-2.25 L and (D) +0.7 to -0.7 A/P and 0.75-2 L.

contralateral responses. In vibrissa responsive sites, contralateral vibrissa movement was often coupled with synchronous stimulation-timed ipsilateral vibrissa movement, consistent with results in rats (Neafsey et al. 1986). Visible stimulation-evoked jaw movements were undetectable in most maps. However, when these movements were amplified by adhering long cat whiskers to the lower jaw of one mouse, the jaw representation was revealed in the lateral region of the head representation area and

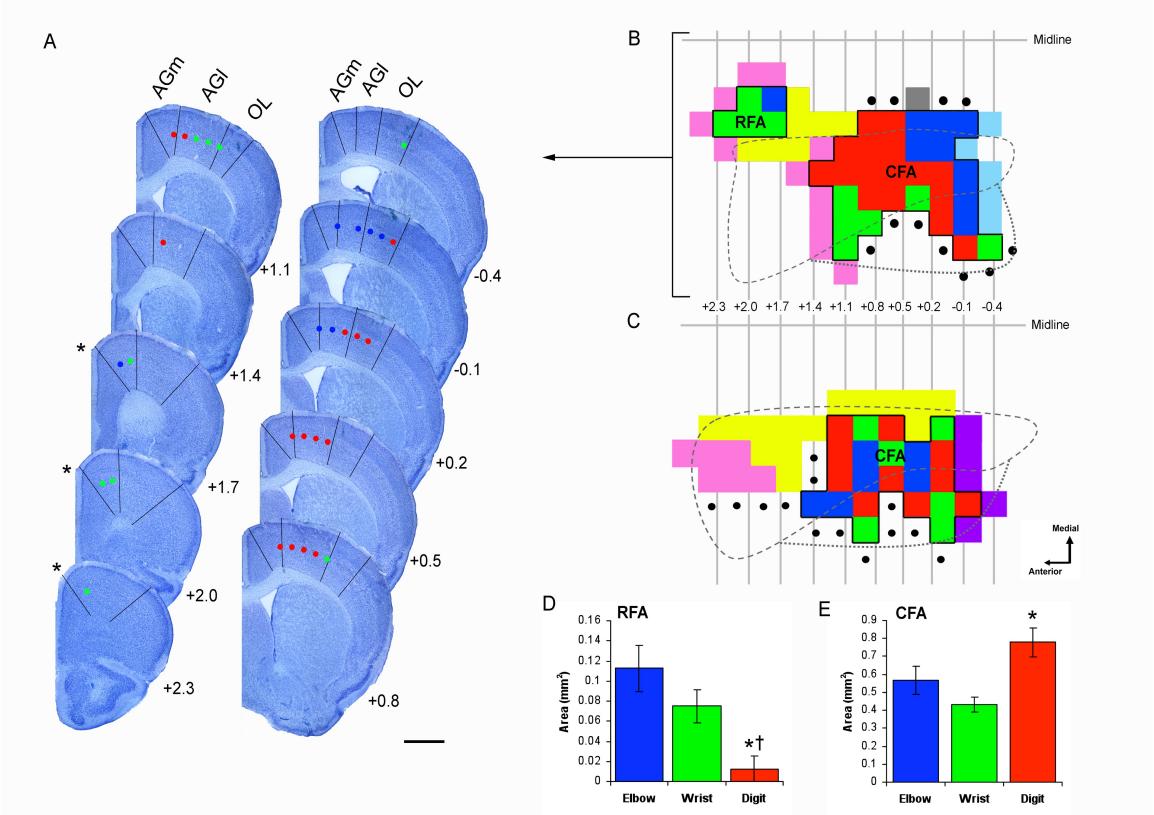


Figure 2.4. *(A)* Representative Nissl stained coronal hemisections showing alignment of forelimb movement representations with cytoarchitecturally defined cortical subregions. Numbers to the right of each section indicate coronal plane relative to bregma. Responsive sites in the three most anterior sections (*) were in rostral forelimb area (RFA) and the remainder were in caudal forelimb area (CFA). Map color codes for movement type match those in Figure 2.2. AGm, medial agranular cortex, AGl, lateral agranular cortex, OL, motor-somatosensory overlap zone. Sections between +2.3 to +1.4 do not contain OL and section +2.3 contains only AGm. Scale bar equals 1 mm. *(B)* Representative forelimb map containing a CFA and RFA. Cytoarchitectonic boundaries for AGl and Ol are delineated as in Figure 2.2. *(C)* A representative forelimb map containing a CFA, but lacking a discernable RFA. The vertical lines indicate coronal section planes and numbers are anterior/posterior distances from bregma (mm). Black dots are nonresponsive sites. *(D-E)* Average areas of elbow, wrist and digit movement representations within the RFA (n = 20) and CFA (n = 27), respectively. Digit representations made up the largest area of the CFA but were rarely found in RFA. The RFA was also much smaller than CFA. (Note the difference in scales and that the RFA data presented here includes only those animals with a discernable RFA.) * $p<0.05$, digit vs. wrist, † $p<0.001$, digit vs. elbow. Data are means ± S.E.M.

comprised 42% of the total head representation (Fig. 2.2H). The RFA, found in 5 out of 6 of this subset of mice, was located anterior to the CFA, separated by neck and/or vibrissa representations (Fig. 2.2C-H). The shoulder representation comprised 7 ± 2 % of the total map area and appeared caudally at the medial and/or lateral borders of the CFA and was distinguishable from the representations of the elbow, wrist, and digit, which were more intermingled in the CFA (Fig. 2.5). The hindlimb representation was in the caudal extent of MI, bordered in the anterior and lateral directions mostly by CFA and/or shoulder, and in the medial and caudal directions by trunk, tail and nonresponsive sites.

2.4.2 Organization of the forelimb representation

A larger number of mice (n=27, including the 6 mice with full MI maps) were used to characterize the forelimb representation area of the motor cortex in more detail (Figs. 2.3-2.5). Like the rat forelimb representation, the mouse representation was separated into two separate forelimb maps in a majority of animals (n=20/27, 74%). The caudal forelimb area (CFA) measured 1.78 ± 0.09 mm^2 in area and comprised 92 ± 1 % of the total forelimb map. The rostral forelimb area (RFA), located anterior to and centered more medially than the CFA (Fig. 2.4B), measured 0.20 ± 0.02 mm^2 in area and comprised the remaining 8 ± 1 % of the total forelimb area. These two areas were usually separated by neck or vibrissa representations. In animals in which no distinct RFA could be resolved, vibrissa and neck movements could be found in the area of the expected RFA (Fig. 2.4C). In these animals, the CFA was similar in total area compared to the CFA of mice that also had a discernable RFA and there was no difference in the rostral

extent of the CFA. There was also no significant correlation between CFA and RFA areal extent (r = 0.23, p = 0.25). It is unlikely that differences in anesthetic plane contributed to the failure to resolve RFA in these animals, as there were no significant differences in the thresholds of elbow, wrist, or digit movements within the CFA of animals with and without a discernable RFA (Fs = 0.32-0.47, ps = 0.57-0.50).

Within the CFA, the digit representation predominated (Fig. 2.4E). Wrist and elbow movement territories each comprised a smaller territory. There was a significant main effect of the areal extent of each forelimb movement type within the CFA (one-way ANOVA for areal extent of movement type: $F(2,78)=6.65$, $p=0.002$). The area of the digit representation, which was 45 ± 4 % of the total forelimb representation, was significantly greater than that of the wrist. The area of elbow representation was not significantly different from either digit or wrist (Fig. 2.4E). Considering the distal forelimb representations together (digit and wrist), these comprised 70 ± 3 % of the CFA. As noted above, the shoulder representation was treated as a separate representation for this analysis. Using data from the full MI maps (n=6), if the shoulder representation is instead considered to be a proximal forelimb representation within the CFA (along with elbow), then distal forelimb representations comprise 51 ± 5 % of the CFA.

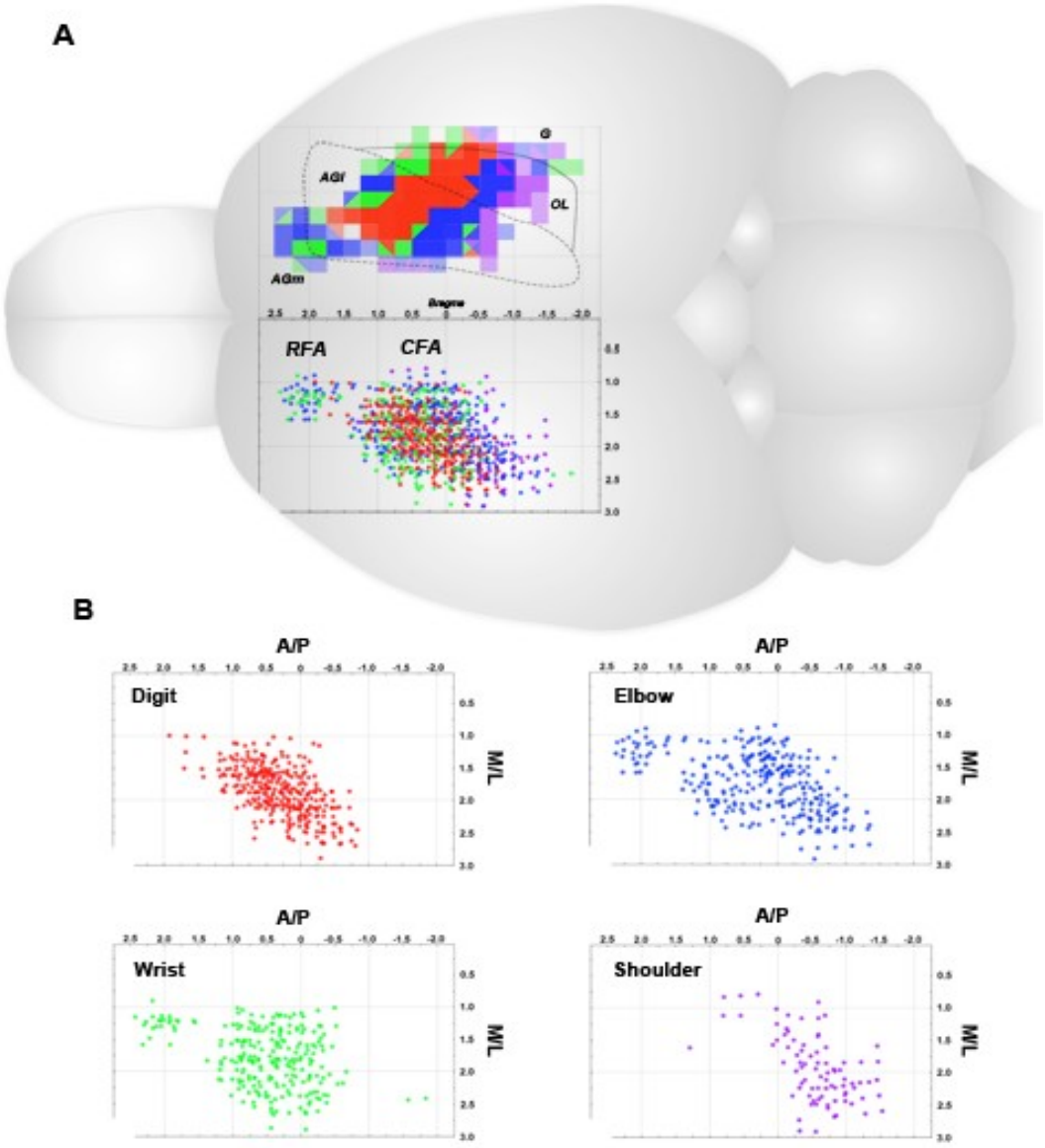


Figure 2.5. Forelimb responsive sites, including shoulder representations, in all mice (n=27) in the study plotted in mm coordinates relative to bregma. (*A)* Forelimb responsive sites on a schematic representation of the mouse cortex (dorsal surface). The right hemisphere (top) shows the modal response per 250 by 250 µm area. Multicolored squares represent movement types tied for the mode. Color intensity represents the frequency of any forelimb response per area. Cytoarchitectural boundaries are indicated as in Figure 2.2. The left hemisphere shows stimulation sites that resulted in forelimb movements in all mice combined. Color codes are the same as those in the graphs in *B. (B)* Separate presentations of the spatial distributions of digit (n=336 sites), wrist (n=187), elbow (n=245) and shoulder (n=82) responsive sites in coordinates (mm) relative to bregma. Map coordinates of individual mice were aligned relative to skull landmarks (bregma and sagittal, frontonasal and lambdoid sutures) and overlaid with transparency. Note that the shoulder representation was not mapped in its entirety in most mice and thus the graphs under-represent its density relative to the other representations.

Unlike the CFA, the RFA was composed of mostly elbow (56 ± 9 %) and wrist (40 ± 9 %) representations, when data from only those animals with a distinguishable RFA are considered. There was very little digit representation, with only 1 animal showing any digit representation within the RFA. There was a significant main effect of areal extent of movement type within the RFA (one-way ANOVA for representation type: $F(2,57)=7.957$, $p=0.001$). Both elbow and wrist representations had significantly greater areal extent than the digit representation (Fig. 2.4D). However, elbow and wrist representations were not significantly different from one another, nor were proximal (elbow) versus distal (wrist and digit) representations.

2.4.3 Relationship of motor maps to cytoarchitecture

The relationship between ICMS-elicited forelimb movements and cytoarchitecturally distinct areas of the sensorimotor cortex was determined by aligning the cortical maps with Nissl stained coronal sections in a subset of animals (n=13; Fig. 2.4A). All forelimb movements were found to be located in one of four cytoarchitectural zones, the AGm, AGl, OL, and G. The area of forelimb movement representations falling in each zone was calculated as the percentage of the total forelimb map. On average, approximately half of all forelimb responsive sites fell into the AGl, one third fell into the OL, and a minimum were in the AGm and G (Fig. 2.6A). Thus, the forelimb representation in the motor cortex of the mouse overlaps with the neighboring region of the granular/sensory cortex to a large extent, as has previously been found in rats (Donoghue and Wise 1982).

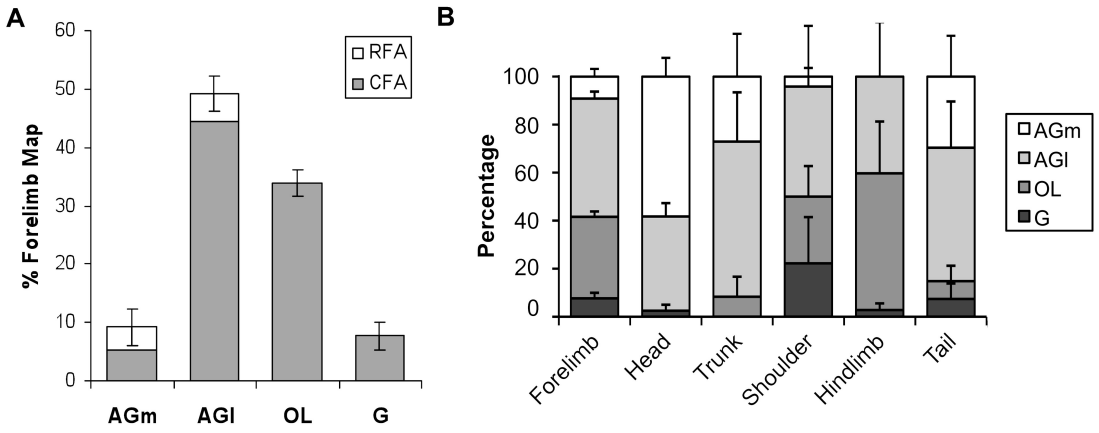


Figure 2.6 Anatomical locations of movement representations as defined by cytoarchitectonics. (A) Percentage of the forelimb maps (n=9) within each cytoarchitectural region. The CFA was primarily located in the cytoarchitecturally defined AGl and OL. The RFA was split approximately equally between AGm and AGl. (B) Percentage of the different movement representations of full MI maps falling into each cytoarchitectural region (n=6). Data are means ± S.E.M.

Though there was a small fraction of the total forelimb map in AGm, it was found to be a prominent territory of the RFA. In animals that had a discernable RFA (n=9), 46 ± 14 % of the RFA was found in AGm and the remainder was found in AGl. No RFA sites were found within OL or G. Furthermore, in these animals, 50 ± 4 % of the CFA was found in AGl and the remainder was in OL (34 ± 2 %), AGm (8 ± 3 %), and G (8 ± 3 %). In animals with only a CFA (n=4), 47 ± 4 % of the CFA was found in AGL and the remainder was in OL (43 ± 2 %), and G (10 ± 4 %). No forelimb sites were found within AGm in mice that lacked a discernable RFA.

In the mice with full-MI maps, rostral movement representations extended into AGm and caudal ones extended more into OL and G (Fig. 2.6B), as would be expected based on their surface coordinates. All movement representations extended through AGl.

Large regions of the head, and smaller portions of the trunk and shoulder representations were also found to extend into AGm. In addition to the forelimb representation, the shoulder, hindlimb and a small portion of trunk representations were found in OL. The tail representation, found in 3 of 4 mice, was found to span the posterior border of the map, mostly within the AGl, but also extended medially into AGm and laterally into OL and G. The shoulder representation had the largest extension into G of all movement representations, and this was nevertheless a relatively small portion of its total territory. Considering all movement representations together, 48 ± 3 % of the motor map was in AGL, 32 ± 3 % was in OL, 12 ± 4 % was in AGm and 7 ± 2 % was in G.

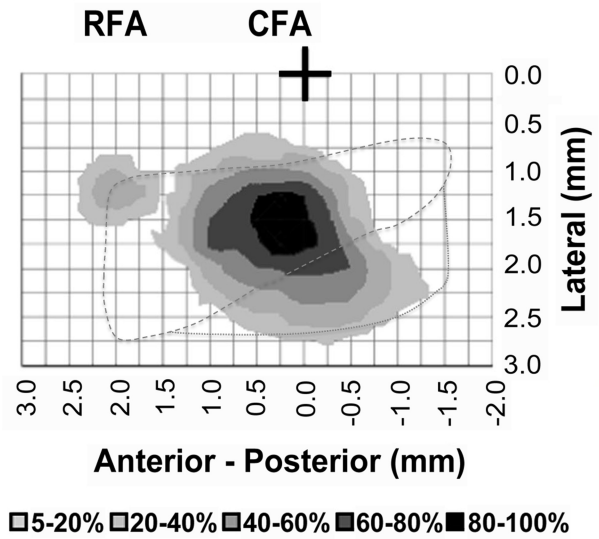
2.4.4 Forelimb motor map distributions in relation to bregma coordinates

To characterize the spatial distribution of the forelimb representation area across animals, a two dimensional frequency distribution of forelimb responsive sites at coordinates relative to bregma was generated (Fig. 2.7). Averaging across all animals of the study (n=27), the forelimb representation was found between approximately 2.5 mm anterior and 1.25 mm posterior to bregma and between 0.75 to 2.75 mm lateral to midline (see also Fig. 2.5). Forelimb movements could be elicited from almost all animals at 0.25 mm anterior and 1.5 mm lateral to bregma, approximately the center of the CFA.

There was no significant difference between the anterior extent (1.09 ± 0.07 mm vs. 0.96 ± 0.12 mm anterior to bregma), the posterior extent (0.64 ± 0.08 mm vs. 0.81 ± 0.20 mm posterior to bregma), or the area (1.85 ± 0.10 mm^2 vs. 1.58 ± 0.17 mm^2) of CFA in animals with and without a discernable RFA, respectively. Thus, the location of the

CFA relative to bregma does not vary greatly in animals with and without an identified RFA.

Figure 2.7 Surface plot showing the frequency distribution of forelimb-responsive sites



(wrist, digit and elbow) at each coordinate relative to bregma (n=27). Nearly all animals had forelimb responsive sites in a region aligned with and extending slightly anterior to bregma in the anterior-posterior direction and approximately 1-2 mm lateral to midline. In most mice, forelimb responses could be elicited within a maximum distance of 2.5 mm anterior to 1.25 mm posterior and 0.5 to 2.75 mm lateral to bregma. The outlines approximate typical boundaries of the AGl (large dashes) and OL (small dashes).

2.4.5 Movement thresholds

Movement thresholds were determined at each responsive site for all maps (Fig. 2.8). Thresholds were defined as the least amount of current that could elicit a visible movement, up to a maximum of 60 μA. The average threshold for all movements observed was 35.00 ± 7.64 μA. The average movement threshold for the CFA (30.08 ± 0.97 μA) did not significantly differ from that of the RFA (29.94 ± 2.21 μA). However, within the CFA, there was a significant main effect of movement type in threshold (one-way ANOVA for movement type: $F(2,78)=10.52$, $p=0.00009$). Digit movement

thresholds were significantly higher than those of either wrist or elbow. Wrist movement thresholds were also significantly greater than those of the elbow. Behavioral experience had no significant effect on thresholds within movement types (untrained/naïve vs. pasta handling/reaching: elbow: $F(3,22)=0.49$, $p=0.69$; wrist: $F(3,23)=0.87$, $p=0.47$; digit: $F(3,23)=1.89$, $p=0.16$). There was no significant difference between the thresholds of each movement type within the RFA. Movement thresholds were similar between mice with and without a discernable RFA. Considering wrist and elbow representations together (because of the almost uniform absence of digit representations in RFA), movement thresholds were 27.74 ± 1.45 μA in mice with a RFA and 27.59 ± 2.24 μA in mice without a discernable one. Consistent with the findings of Li and Waters (1991),

Because the strategy of the present study was to minimize the stimulation needed to reveal maps (so as to minimize stimulation effects on the maps; Nudo et al. 1990), it is not amenable to characterizing dual response areas or movement sequences, as done in other studies (Neafsey et al. 1986; Ramanathan et al. 2006). That is, as soon as a movement was observed, the intensity was lowered to establish thresholds. However, even with this approach, multiple movements were frequently initially elicited at stimulation levels above the movement threshold, but below the maximum level of stimulation (60 μA). Although these movements were not systematically recorded, anecdotally there were clear patterns in the movement combinations found together as well as patterns of movements found near borders versus the center of representations, consistent with previous observations in rats (Neafsey et al. 1986). Forelimb movements were observed with non-forelimb movements, especially vibrissa, neck, and hindlimb

near the borders of the forelimb area and these respective representations. Movements of digit, wrist and/or elbow were also often observed together. The dual responses tended to be of the wrist and digit (with digit alone at lower thresholds), or of the elbow and wrist (with wrist alone at lower thresholds). A dual response of elbow and digit without a simultaneous wrist movement was never seen.

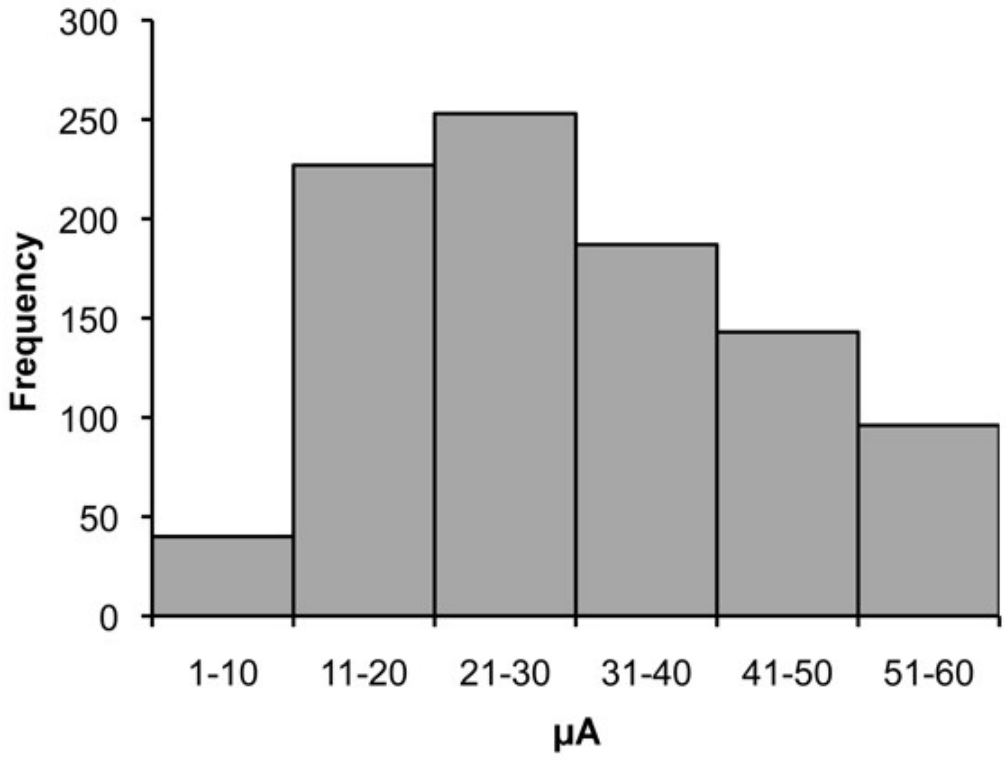


Figure 2.8 Bar graph of the frequency of movement thresholds in 10 µA bins for all forelimb movements in all maps (946 responsive sites summed across n = 27 maps).

Data from the full MI maps (n=6) comparing neck/jaw movement thresholds between animals that had a vibrissa representation (n=2) and animals that did not (n=4) show that there is a tendency for neck thresholds to be higher in those animals lacking a vibrissa representation (30.69 ± 1.34 μA vs. 25.05 ± 2.21 μA, $t(116) = 1.89$, $p = 0.06$), suggesting that anesthesia differences may influence the resolution of vibrissa representation, as has been found in rats (Tandon et al. 2008).

2.5 Discussion

Using intracortical microstimulation (ICMS) and cytoarchitectural analyses to characterize the mouse motor map, we found that the C57BL/6 mouse has a motor map that resembles, in its general organization, those found in other species, such as rats (Donoghue and Wise 1982), cats (Asanuma and Sakata 1967), and monkeys (Nudo et al. 1996). We also found that C57BL/6 mice share several specific characteristics with rats, as discussed below. Furthermore, although there are some notable differences, as detailed below, our results generally agree with the ICMS generated motor maps described in other strains of mice (Li and Waters 1991; Pronichev and Lenkov 1998), in that we found a relatively large forelimb representation in the motor cortex, from which we were able to elicit movements of the elbow, wrist, and digit. Although both previous mouse ICMS studies comment on the observation of elbow, wrist, and digit movements, we are the first to quantify the areal extent of these representations. Such characterization is important for the study of motor cortical plasticity because the overall map area can remain unchanged while there is internal reorganization of the movement subtypes (e.g.,

enlargement of distal at the expense of proximal forelimb representations; Kleim et al. 1998).

Like rats, we found that a majority of animals have two distinct forelimb representations: a large caudal map (CFA) and a smaller rostral map (RFA). Although the function of the rodent RFA remains poorly understood, it has been proposed that the RFA of rats may be homologous to the premotor or supplementary motor areas of the primate brain (Neafsey and Sievert 1982; Barth et al. 1990; Rouiller et al. 1993; Dancause et al. 2006; Eisner-Janowicz et al. 2008). Given the similarities between the mouse and rat RFA in relative size and location, these functional distinctions and homologies may extend to the mouse brain as well. From the surround of the forelimb area we were able to elicit movements of the vibrissae, neck, jaw, shoulder, trunk, hindlimb, and tail. This organization parallels that of the rat motor cortex, although a greater proportion of the mouse CFA seems to extend posterior to bregma, compared to the rat CFA (Fig. 2.7). In our approach, as in prior rat studies (Kleim et al. 1998, 2002, 2004), borders of movement representations are established first followed by delineation of the interior, because repetitive stimulation of the borders of the map can cause them to expand (Nudo et al. 1990). We cannot rule out the possibility that movement representations exist outside of the nonresponsive borders. However, movement representations tend to be organized in islands of individual body part representations that appose (and often overlap with) surrounding representations (e.g., Neafsey et al. 1986) and it is unlikely that the approach missed a large expanse of any of the movement representations studied.

The most striking difference in the forelimb motor map of mice compared with rats is the prominence and location of the digit representation. The digit representation in the mouse motor cortex is large, whereas digit movements are rarely evoked with ICMS in the rat motor cortex (Hall and Lindholm 1974; Donoghue and Wise 1982; Kleim et al. 1998) and when they are, they are usually found in the RFA, and rarely in the CFA (Kleim et al. 1998). In mice, we found that significantly more digit movements than either elbow or wrist movements were elicited from the CFA, while the RFA was mostly composed of elbow and wrist representations. We also found that the movement thresholds of digits were higher than those of wrist and elbow, despite the greater prevalence of digit responsive sites. The pattern of threshold differences in rats is opposite of what we have found in mice, such that elbow movements are evoked at higher thresholds and digit movements at lower thresholds, even though digit responsive sites are rare in rats (Kleim et al. 1998). Spider monkeys naïve to behavioral manipulations have no significant differences in the movement thresholds of digit, wrist, elbow, and shoulder movements (Donoghue et al. 1992). However, when monkeys are trained on a skilled reaching task, there is a significant *increase* in the thresholds of the trained movement, concurrent with areal expansion of digit representations (Nudo et al. 1996). Additionally, Nudo et al. found that map size is not determined by movement thresholds because the areal expansion was evident even when maps were generated at a fixed stimulation current.

The area of the map devoted to a movement is thought to correspond with the dexterity of that movement (Monfils et al. 2005) and, thus, it is tempting to speculate that

the difference in digit representations corresponds to species-related differences in manual dexterity. Large digit maps of monkeys (Nudo et al. 1996) and humans (Kim et al. 2004; Schwenkreis et al. 2007) have been related to their manual dexterity (reviewed in Monfils et al. 2005). Furthermore, the CFA, where digit representations predominated in mice, may have a more essential role in skilled reaching than the RFA (Gharbawie et al. 2007). Though the present study intentionally included mice with variability in behavioral experiences to broaden reproducibility (Richter et al., 2010), the experimenter-controlled experiences had no influence on the ICMS measures. This raises the possibility that mice are spontaneously more engaged in dexterous activity with the distal forepaws than are rats. However, this idea seems at odds with the behavioral characterization of rats as being extremely dexterous with their forepaws, including their ability to manipulate food items with skillful and independent movements of the digits (Whishaw and Gorny 1994; Whishaw and Coles 1996; Allred et al. 2008). Though mice are beginning to be used more for studies of forepaw skill learning and dexterity (Farr et al. 2006; Tennant and Jones 2009; Xu et al. 2009) and recovery of function after brain injury (Menalled and Chesselet 2002; Carmichael 2005; Fleming and Chesselet 2006; Horie et al. 2008; Brown et al. 2009; Xiong et al. 2010), their forepaw behaviors have not been as well characterized as that of rats.

Forepaw dexterity in rats has been related to the total area of the distal movement representations (wrist and digit) at the expense of proximal (elbow and shoulder) movement representations. In untrained rats, approximately 40% of the forelimb map is comprised of distal forelimb representation whereas, after substantial training in a skilled

reaching task, the distal representation comprises approximately 75% of the rat forelimb map (Kleim et al. 1998). Although we did not include shoulder movements in our detailed characterization of the forelimb representation in all of the mice, in the subset of mice (all naïve) in which it was included, the forelimb representation was split almost equally between distal (wrist and digit) and proximal (elbow and shoulder) representations (~51 and 49%, respectively). Thus, though the large digit representation of the mouse is remarkable compared with that of the rat, the total proportion of the distal forelimb representation of naïve mice better approximates that of an unskilled, than a skilled rat. Thus, if species-specific forelimb behaviors are linked with the variations in motor maps, they might reflect variations in movement manner rather than degree of dexterity. Furthermore, it cannot be ruled out that the same ICMS parameters and anesthetics result in different neural activating effects across these species and that this contributes to a more sensitive detection of digit representations in the mouse compared with the rat motor cortex.

We also related the mouse motor cortex to the underlying cytoarchitecture. We found that the majority of the motor map was found in the lateral agranular cortex (AGl), but it extended into adjacent cytoarchitecturally distinct regions. The most prevalent location of forelimb representations was in the AGl, but approximately half of the RFA was in the AGm and approximately half of the CFA was in the primary motor and sensory overlap zone (OL). Based on cytoarchitectural and ICMS studies of rats (Donoghue and Wise 1982; Kleim et al. 1998), the RFA has approximately the same placement relative to cytoarchitecture in both rats and mice. In rats, the posterolateral

portion of the CFA is known to overlap with the anteromedial portion of the forelimb somatosensory map (Hall and Lindholm 1974; Donoghue and Wise 1982). This area is cytoarchitecturally distinct from either primary motor cortex or primary somatosensory cortical areas, as it contains both a distinct layer IV with densely packed granular cells and layer V characteristics of AGl (Donoghue and Wise 1982). In mice, this cytoarchitectonic pattern was clearly distinguishable in Nissl stained coronal sections. This pattern was also described by Caviness (1975) as characteristic of the interface of frontal and parietal cortex in mice. Our finding that approximately half of the CFA and hindlimb areas fall into the overlap zone, as cytoarchitectonically defined, supports the idea that there is an overlap between primary motor and somatosensory cortices in the mouse brain. Using intrinsic optical signal (IOS) imaging of sensory cortex combined with light-based mapping of motor cortex, Ayling et al. (2009) also found that approximately half of the forelimb and hindlimb motor maps overlapped with their respective sensory maps. Combined with our results, this lends support to the idea that the forelimb cortex has an overlap zone similar to that seen in rats, and that the hindlimb motor and sensory maps do not overlap completely in mice, as they do in rats (Hall and Lindholm 1974; Donoghue and Wise 1982). However, this finding needs to be verified using electrophysiological recording from somatosensory cortex.

Another notable variation from the rat motor map was in the vibrissa representation. The rat vibrissa representation extends along the medial and anterior aspect of the motor cortex. The mouse vibrissa representation follows this same pattern, but was also found to extend along the lateral aspect of the forelimb area, thus wrapping

around the rostral borders of the motor map (Fig. 2.2E-F). This characteristic was evident both in the full MI maps and in the borders of forelimb-only maps (Figs. 2.3, 2.4). However, the absolute areas of individual movement representations of the head could not be rigorously analyzed in this study because the ICMS protocols for sensitively distinguishing vibrissa and jaw representations from those of the neck in mice require further refinement. Tandon et al. (2008) showed that the vibrissa and neck representations in the rat motor map are highly sensitive to minor variations in anesthetic depth, while the representation of the forelimb is relatively insensitive to the same range of anesthesia depths. Thus, although we were able to resolve a complete map of the forelimb in all mice, the 11/27 mice lacking vibrissa responsive sites may have been in a plane of anesthesia that was not optimal for resolution of the vibrissa representation. However, the manifestation of the optimal anesthetic plane for resolving the vibrissa representation still needs to be established in the mouse. Also, the methods for identification of jaw movements used in rat ICMS (visible stimulation-timed movements of the jaw) may fail to detect most jaw movements in the mouse because they are too subtle to discern in this manner. In anecdotal support for this possibility, visually amplifying the jaw movement (by adhering cat whiskers to the chin) revealed a substantial jaw representation in one mouse that was not found in any other mouse mapped without cat whiskers.

The stimulation protocol we used differed in some parameters from those used in earlier mouse mapping studies but was the same as those used by our group and others to characterize the organization of the rat motor cortex (Kleim et al. 1998; Gharbawie et al. 2005; Markham et al. 2007; Young et al. 2009). We chose to do this in order to facilitate

interspecies comparisons of motor cortical organization. Our anesthetic and stimulation patterns, as well as the resulting map areas, were more similar to those used by Li and Waters (1991) than to those used by Pronichev and Lenkov (1998). Pronichev and Lenkov (under thiopental anesthesia) did not stimulate above 40 μA, and their thresholds for forelimb responses ranged from 10-35 μA. Li and Waters found many forelimb responses under ketamine anesthesia at thresholds between 26-50 μA. Our forelimb movement thresholds (under ketamine anesthesia) ranged from 5-60 μA (Fig. 2.8). Also, it is possible that Pronichev and Lenkov's approach was less sensitive to the presence of RFA due to the relatively wide grid spacing (500 μm) between stimulation sites, as RFA is typically limited to only a few points even when mapped in a 250 μm grid. Ayling et al. (2009) and Hira et al. (2009) also did not report the presence of a separate RFA in the channelrhodopsin-2 mice mapped with light stimulation. Although both groups monitored forelimb and hindlimb responses, they did not distinguish between different types of forelimb movements or monitor other body movements. It could be possible to overlook the distinction between CFA and RFA because neck or vibrissa are found at the lowest stimulation thresholds in the cortical region between them, but these responses often can be elicited together with forelimb movements at higher stimulation intensities. Thus, in these, as well as the present study, it should not be assumed that RFA is non-existent in these animals, because it may instead simply be difficult to distinguish from the surrounding, overlapping representations of the head.

Our results for map location, size, and the existence of two distinct forelimb areas resemble the results of Li and Waters (1991), so it appears that the dystrophic strain of

mice they studied does not dramatically differ in these parameters compared with the wildtype C57BL6 mice of our study. In humans with muscular dystrophy, there is no major deficit in the ability to elicit movements using transcranial magnetic stimulation, although the evoked movements are smaller in amplitude (Yayla et al. 2008) and are elicited at higher threshold currents (Di Lazzaro et al. 1998). In comparison to our results, Li and Waters' representative motor maps contained less digit representation, particularly in the CFA, but they did not report areal extents of individual movement representations. If the dystrophic mice do have on average smaller digit representations, and if higher threshold currents are required to elicit movement in these animals, it is possible that more digit representations would have been revealed with higher stimulation intensities because we found that movement thresholds for digit representations are higher than those of wrist and elbow.

In summary, our findings indicate that the mouse motor cortex has general similarities to the rat motor cortex, including its relative size, location to other movement representations, and the existence of two distinct forelimb areas. However, the mouse motor cortex has significantly more digit representation relative to wrist or elbow representation, which is not seen in the rat motor cortex, even after skilled motor training. Additional work is needed to more fully characterize the overlap zone of the CFA, to characterize head representations in more detail, and to relate the motor map organization to corticofugal projection patterns.

Chapter 3: Teaching old maps new tricks: Age-related differences in organization of the forelimb motor cortical representation and its response to motor skill learning

3.1 Abstract

Movement representation areas in the motor cortex have been shown to reorganize to support motor skill learning during young adulthood. However, there is little known about how the motor representation changes with age and how this relates to the loss of dexterity associated with aging. This study used intracortical microstimulation to characterize the organization of the forelimb motor cortex in young and aged mice, with and without training of the forelimb on a skilled reaching or a dexterous food handling task. Our results indicate that the ability to learn a new skilled motor task was maintained in old age, despite an age-associated loss in digit representation area in motor cortex. In young mice, a short duration of behavioral training caused an increase in proximal movement representations at the expense of distal movement representations. Following a longer period of practice, motor map organization returned to control levels. This pattern of plasticity was not seen in aged mice, suggesting that the plasticity of motor cortical representations is altered in old age.

3.2 Introduction

Aging is a major risk factor for accidental injury related to declining motor function. One third of adults over the age of 65 fall each year (Hausdorff et al, 2001). The

upper extremities often play a critical role in helping to “break” the fall and prevent injury (Sran et al., 2010). Thus, age-related decreases in dexterity of the hands and digits may result in an increased risk of injury as well as trouble performing tasks of daily living. This leads to a loss of independence and may also be an early predictor of cognitive decline (Kluger et al., 1997). However, in healthy subjects, the loss of dexterity associated with aging can be attenuated. Skilled training on a finger movement task improved the ability of older adults to control pinch force, hand steadiness, and manual speed, compared to untrained older adults (Ranganathan et al., 2001). A better understanding of the differences between young and aged forelimb motor function and organization of the motor cortex in healthy individuals could lead to the development of strategies to extend youth-like motor ability and manual dexterity into old age.

Age-related changes in motor performance are associated with alterations in the function of the motor systems in the brain. Transcranial magnetic stimulation (TMS) and functional magnetic resonance imaging (fMRI) studies indicate greater activation of premotor, supplementary motor, sensory and cognitive areas in the aged brain during motor task performance (Hutchinson et al., 2002; Ward and Frackowiak, 2003; Heuninckx et al. 2005, 2008; Talelli et al., 2008). The increase in cognitive and sensory activation is thought to reflect an increased reliance on sensory cues and more cognitive control during movement, which may allow older adults to produce motor behavior that is more similar to that seen in young adulthood (Heuninckx et al. 2005, 2008).

Due to their dexterous use of the forepaws during sensorimotor tasks, rats and mice provide useful models for studying loss of forelimb dexterity during aging. The

topographical complexity of the forelimb somatosensory cortical representation of rats degrades as they age (Coq and Xerri, 2000) and this coincides with impairments in walking behavior (David-Jürgens et al., 2008). The somatosensory map regains some complexity (Coq and Xerri, 2001) and walking behavior improves (David-Jürgens et al., 2008) when rats are housed in enriched environments from weaning. In contrast to the somatosensory cortex, little is known about how the organization and plasticity of the rodent motor cortex changes in old age. In young adult rats, Kleim et al. (1998) found that training on a skilled reaching task results in an increase in representation areas for movements necessary for task performance at the expense of representations of lesser-used movements. In young adult mice, dendrites in the motor cortex begin to add new spines within the first hour after onset of training on a skilled reaching task. The total spine density returns to baseline when a skill has been practiced for ~ 2 weeks, but there is selective survival of new spines formed during early skill acquisition (Xu et al., 2009). Molina-Luna et al. (2008) have previously shown that the areal extent of the forelimb movement representations in rats increases during acquisition of a motor skill and returns to baseline after 8 days without training, but the ability to perform the task is maintained. It is unknown why the map returns to baseline without a loss of motor skill, and what effect different types of training (e.g., greater intensity, longer duration/extended practice) may have on the reorganization of the motor map. Thus, more information is needed on the specific conditions under which motor cortical plasticity occurs, even in young adult brains, and how these changes relate to alterations in motor behavior throughout the lifespan.

The purpose of the current study was to extend these findings of young adult cortical plasticity by investigating how the mouse forelimb motor representation reorganizes in response to variations in the duration and intensity of motor skill learning and how sensorimotor behaviors and the organization of motor cortical movement representations change with age. Our results indicate that the reorganization of movement representations depends on the duration, but not necessarily the intensity, of motor training. Additionally, the organization and plasticity of the forelimb motor cortical representation is altered in the aged brain, but the ability to learn a skilled motor task is maintained.

3.3 Methods

3.3.1 Subjects

A total of 91 young (3-5 months old) and 43 aged (18-20 months old) well-handled male C57BL/6 mice were used. All young mice were purchased from Jackson Laboratories (Bar Harbor, ME). Aged mice were either retired breeders obtained from the Transgenic Mouse Facility at UT-Austin or Jackson Laboratories (n = 26), or virgin mice obtained from Jackson Laboratories at 1 month of age and allowed to age in our colony (n =17). The two groups of aged mice did not differ significantly in behavioral or ICMS evoked motor map results and were combined for all analyses. Young mice and aged mice raised in our colony were housed in groups of four. Aged retired breeders were housed singly before arrival in our colony, and were maintained in single housing to prevent aggressive behavior characteristic of non-littermate males. All mice received

standardized housing supplementation (a small piece of PVC pipe, a cardboard roll, small wooden objects, and nesting material) and were kept on a 12:12 h light/dark cycle. Animals were maintained on scheduled feeding, given at the end of each training day to prevent satiation at the time of reach training. Aged animals received slightly more food (3-3.5 g/day) than young animals (2.5-3 g/day) to account for their larger size (30.8 ± 0.6 versus 28.4 ± 0.2 g in young mice). A total of 6 animals (4 young and 2 aged) died during the ICMS procedure before a complete forelimb map was resolved. Behavioral data from these animals are included in the study. ICMS data from 27 of the young animals were included in a previously published paper describing the general organization of the mouse motor cortex (Tennant et al., 2011; Chapter 2), and some data from the subset of animals tested on the Capellini Handling Test were included in a previously published methods article (Tennant et al., 2010b). Animal use was in accordance with a protocol approved by the University of Texas at Austin Animal Care and Use Committee.

3.3.2 Design of behavioral experiments

Both young and aged animals were randomly assigned to one of three training conditions (Fig 3.1): Reach Training, Pasta Handling, or Untrained Control conditions and one of two duration conditions: Short Duration (2-4 weeks) or Long Duration (8 weeks). Mice were trained in skilled reaching on the Pasta Matrix Reaching Task (Ballermann et al., 2001; Tennant and Jones, 2009). The Pasta Matrix Reaching Task is similar to the skillful reach-to-grasp tasks that have previously been shown to reorganize motor representations in the rat (Kleim et al., 1998). However, it differs in its requirement

to also establish mastery in handling the retrieved food (capellini pasta). Because experience in pasta handling has been found to result in spine plasticity in the motor cortex (Xu et al., 2009), we additionally examined groups trained on pasta handling alone. The different durations of training allowed us to determine if short and long-term practice of a motor skill resulted in differences in reorganization of the forelimb motor cortical representations. Additionally, because the intensity training group received approximately the same total number of reaching sessions as long-duration trained mice, analysis of this group's results provided information on whether the total number of reaching trials or the time span of reach training over days had a greater effect on motor map plasticity.

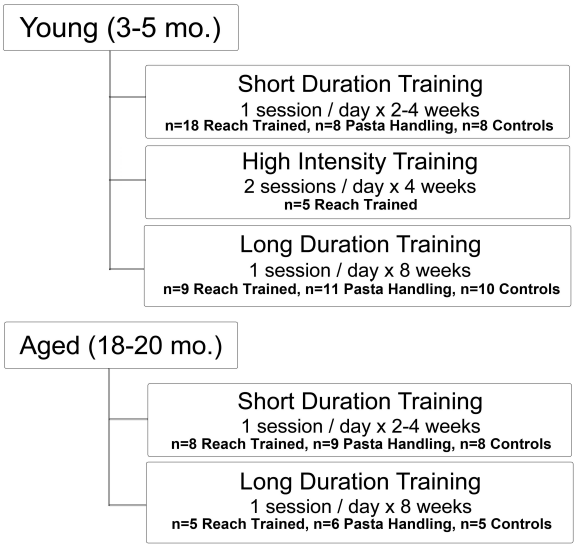


Figure 3.1 Schematic of the training conditions used in the current study. ICMS mapping of motor cortex was conducted 1-2 days after the last training (or control) session with the exception of the short-duration delayed map condition (n=8 of the reach trained group) which underwent ICMS mapping 4 weeks after the end of training.

Equal numbers of mice from each behavioral condition were trained together to control for batch effects. The duration of training was determined in individual mice depending on when they transitioned from rapid to gradual improvements in performance, approaching asymptotic performance. Short-duration mice were trained until they retained approximately asymptotic performance for at least 7 and no more than 14 days. Long-duration mice were trained until asymptotic performance was retained for ~45 days. Two additional reach training conditions were examined in young mice only: a High Intensity condition in which animals were trained twice daily for a short duration and a Delayed Map condition, in which animals were trained for a short duration (4 weeks) but mapped after a delay of 4 weeks to match the long duration schedule (8 weeks).

A subset of mice in the short-duration training conditions (n=8 young, 7 aged) were additionally tested at once-weekly intervals on the Ladder Rung Walking Test (Metz and Whishaw, 2002; Farr et al., 2006; Tennant and Jones, 2009), the Bilateral Tactile Stimulation Test (Schallert et al., 1982; Schallert and Whishaw, 1984; Tennant et al., 2009), and the Capellini Handling Test (Allred et al., 2008; Xu et al., 2009; Tennant et al., 2010b). Animals were tested on multiple occasions to control for week-to-week variability of sensorimotor behavior. Additionally, because practice on the Capellini Handling Test has previously been shown to elicit plasticity in the motor cortex (Xu et al., 2009), only animals from pasta handling and reach training conditions were tested on the sensorimotor battery so these groups were similarly practiced in pasta handling and any additional learning effects would not confound the ICMS results of control animals.

However, the limited testing on this sensorimotor battery had no discernable influence on reorganization of motor representations, as determined by comparisons within training conditions of mice that did or did not receive the test battery (*t*s= -1.02-0.40, *p*s=0.17-0.65).

3.3.3 Motor skill training tasks

Daily training on the Pasta Matrix Reaching Task involved training mice to reach for and break small pieces of vertically oriented, uncooked capellini pasta (3.2 cm in height and 1 mm diameter; DeCecco brand, Fratelli De Cecco di Filippo Fara San Martino S.p.A., Italy), arranged in a matrix distal and lateral to the reaching chamber aperture (Tennant and Jones, 2009). In order to successfully retrieve a pasta piece, the mouse must break the pasta by grasping and pulling forward. Mice first underwent a short shaping period in order to determine limb preference. Mice were then trained to reach only with the preferred limb by filling with pasta only the half of the matrix contralateral to this limb. Daily training sessions consisted of up to 100 reaches or 15 min, whichever occurred first. Recorded data included the number of pasta pieces successfully broken, the total number of reach attempts, the area of the matrix that the mouse cleared of pasta, the time taken to make 100 reach attempts (to a maximum of 15 min.), and the number of days until asymptotic performance was reached.

To control for effects of training environment across conditions, daily Pasta Handling training consisted of animals being placed into a reaching chamber with no Pasta Matrix placed in front of the reaching aperture. At the same time, another mouse

was being trained on the Pasta Matrix Reaching Task. Pasta Handling animals were yoked to this mouse, receiving the same number of pasta pieces and remaining in the testing chamber for the same amount of time. No data on pasta handling was collected during daily training sessions, but a more detailed analysis of pasta handling behavior, the Capellini Handling Test, described below, was conducted on the subset of mice tested on the sensorimotor battery.

3.3.4 Capellini Handling Test

Mice were placed into a Plexiglas cylinder on top of a mirrored surface, and were videotaped while eating 3-4 pieces of 2.6 cm long uncooked capellini pasta at an angle that permitted view of both paws in the mirror. Slow-motion video analysis was used to measure the total time to eat each pasta piece and the number of adjustments made with each paw. Mice eat the pasta by moving the pasta piece into the mouth with the paws, which are typically used in an asymmetrical manner with one limb placed near the mouse (the guide limb) and the other holding the pasta further down the piece in a full-paw grasp (the grasp paw). An adjustment was counted each time the mouse's paw or digits released and regrasped the pasta piece. Particular attention was paid to the number of "superfluous adjustments", defined as adjustments that did not result in advancement of the pasta piece into the mouse. Often, these appear as repeated fluttering movements of the digits, a behavior that increases during aging (Tennant et al., 2010b). Other atypical eating patterns that were counted included dropping the pasta piece during eating, keeping the paws together when the pasta piece is long, or apart when the pasta piece is

short, switching the positions of the guide and grasp limbs, and failing to contact the pasta with one paw. Atypical eating patterns are described in more detail by Allred et al. (2008) and Tennant et al. (2010b). The mean time to eat, mean number of adjustments with each paw, and mean number of atypical behaviors were calculated by averaging across trials.

3.3.5 Ladder Rung Test

Mice were videotaped while walking across an elevated horizontally oriented ladder. The home cage was placed at one end of the ladder and the mouse was placed at the other end and allowed to walk across the ladder and into the home cage (Farr et al., 2006; Tennant and Jones, 2009). Three ladder crossings per testing day were videotaped. Slow-motion video analysis was used to count incidences of paw slips between or off a rung (errors) and the total number of steps with each paw. The number of errors per crossing, the total number of steps, the number of errors per step, and the average step length (in rungs) were calculated.

3.3.6 Bilateral Tactile Stimulation Test

The mouse was placed into a shallow transparent plastic container with an open top and allowed to habituate for 1 min. The mouse was picked up and lightly restrained by the scruff while a ~1.25 cm long piece of 3 mm wide tape (crepe art tape, Office Depot, Delray Beach, FL) was placed onto the ventral side of each paw. The mouse was then placed back into the container and allowed to remove each piece of tape using its

mouth (Schallert et al., 1982; Schallert & Whishaw, 1984; Tennant and Jones, 2009). The latency to contact and remove each piece of tape was recorded for five trials per day, allowing 30 seconds of rest between each trial. The removal time for each stimulus was calculated by subtracting the contact time from the total removal time in order to separate the motor component from the sensory component of the test.

3.3.7 Intracortical microstimulation (ICMS) mapping

Detailed methods of the ICMS procedure are described in Chapter 2. Animals were anesthetized with an initial cocktail of ketamine (150 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) that was supplemented with additional ketamine and isofluorane (0.5-1 % in oxygen) as necessary. The mouse was placed into a mouse stereotaxic frame (Stoelting, Wood Dale, IL), lidocaine (2 mg/kg, s.c.) was injected into the scalp, and a midline incision was made. The cisterna magna was punctured to drain cerebrospinal fluid (which reduces cortical upwelling), and the skull and dura overlying the motor cortex were removed. The craniotomy was then filled with warm (37° C) silicone oil to prevent drying. A picture of the cortical surface was taken and overlaid in Canvas software with a grid consisting of 250 X 250 μm grid intersection spacings. Sites of stimulation were at grid intersections with the exception of minor adjustments in placement needed to avoid penetrating surface vasculature. Intracortical penetrations with a glass microelectrode (tip diameter of 20-25 μm) with a platinum wire were made using a hydraulic micropositioner at a depth of 790-800 μm (corresponding to deep layer V). Penetrations were throughout the cortex until the entire extent of the forelimb representation was resolved, including

the caudal and rostral aspects of the forelimb motor map. In animals trained for a short duration, shoulder was not included as part of the forelimb during ICMS experiments. However, because the shoulder may be activated during reaching, we began to map the shoulder representation in its entirety for the long-duration trained animals. The absolute areas of shoulder representations of these animals are included in Table A2. At each site, a 40 ms train of 13 200 μs monophasic cathodal pulses was delivered at 350 Hz from an electrically isolated, constant current stimulator (BAK Electronics, Mount Airy, MD) at a rate of 1 Hz. Stimulation was increased up to a maximum of 60 μA, or until a visible movement was evoked. If a movement was evoked at or below 60 μA, the threshold current was determined by gradually decreasing the stimulation until the movement stopped. The lowest amount of stimulation required to evoke movement was determined to be the threshold current. If no movement was seen at 60 μA, the site was considered non-responsive. In cases where stimulation evoked more than one movement, the site was considered responsive to the movement that was determined to have the lowest threshold. Electrode penetrations were made in a systematic manner across the cortex until the entire extent of the caudal and rostral aspects of the forelimb map were resolved. At the end of the ICMS procedure, animals were euthanized with an overdose of sodium pentobarbital (175 mg/kg, i.p.). The areal extent of individual movement representations was calculated by multiplying the number of stimulation sites producing a given movement by the area of a grid square (0.0625 mm^2).

3.3.8 Statistics

All statistical analyses were conducted using SPSS software. To test for age and training condition effects in performance over time on the Pasta Matrix Reaching Task, we performed repeated-measures analyses of variance (ANOVAs) over days of training to test whether reaching performance was affected by age and intensity of training. Age x training day effects were tested in two analyses: one inclusive of all but the high intensity training condition was performed for training days 1-14 (i.e., the short duration training period). A separate ANOVA inclusive of long duration groups was performed for training days 15-60 (the long duration training period). The effects of intensity x training day were tested in an ANOVA that included only young short duration groups (standard vs. high intensity groups) for days 1-14. For the non-repeated measure of days until asymptote, two-tailed t-tests were performed to determine the effects of age (young vs. aged) and intensity of training (young vs. young high intensity). For analysis of final success, age x condition effects were tested in a univariate ANOVA to determine effects of age and duration of training. A t-test was conducted to determine the effect of intensity of training on final success (young short duration vs. young high intensity). Two-tailed t-tests were also used to compare performance between young and aged animals on the Ladder Rung Task, the Bilateral Tactile Stimulation Task, and the Capellini Handling Task, with performance averaged over time.

To test for age and training condition effects on reorganization of forelimb representations, we conducted *a priori* planned comparisons on ICMS data collected from the CFA and RFA of young and aged mice. Planned comparisons were designed to

test whether the ratio of proximal (elbow) to distal (wrist and digit) forelimb representations was affected by 1.) short duration reach training (controls vs. both short duration reach trained groups: standard and high intensity; controls vs. only standard intensity short duration) or 2.) continued practice for a long duration (short duration vs. long duration; controls vs. long duration). In long duration reach trained animals, full shoulder representations were mapped and two separate analysis were done. In the first analysis, the proximal forelimb area included only elbow, as in the short duration analyses. In the second analysis, the proximal forelimb area included both elbow and shoulder representations. These comparisons were conducted separately for young and aged animals. In addition, we performed planned comparisons of data from young mice to determine if proximal to distal forelimb ratios were affected by 1.) the intensity of training (low intensity short duration vs. high intensity short duration) or 2.) the time between cessation of training and ICMS data collection (controls or short duration vs. delayed maps). To additionally determine if pasta handling experience had an effect on the forelimb motor cortical representations, we conducted an ANOVA comparing all pasta handlers (short and long duration) vs. all controls. If so, we used a secondary analysis to determine the effect of pasta handling duration (short pasta handlers vs. long pasta handlers). Two-tailed t-tests were performed to compare movement thresholds.

3.4 Results

3.4.1 Effect of age on acquisition and performance of motor tasks

3.4.1.1 Pasta Matrix Reaching Task

To determine how the motor map reorganizes in response to motor skill learning and extended practice throughout the adult lifespan, we trained young and aged mice on a skilled reaching task, altering the duration and intensity of daily reaching sessions (Fig. 3.1). The Pasta Matrix Reaching task was chosen because this task involves the development of novel, skillful movement sequences of the forelimb. In order to successfully perform the task, mice learn to break and retrieve small pieces of vertically oriented dry capellini pasta arranged in a matrix placed directly outside of a slit in a Plexiglas reaching chamber. The learning curve on this task involves an early rapid learning phase characterized by a steep learning curve. As training progresses, the learning improvements become more gradual and the learning curve becomes relatively flat. We found that young and aged mice had similar rates of learning as defined by the reaching success over days of training, the number of days until the early rapid learning phase became more gradual, and the final level of success following short and long-duration training (Fig. 3.2).

While young and aged animals initially had similar rates of learning, aged animals surpassed the success rate of young animals with ongoing training (Fig. 3.2A). During the short duration portion of training, there was a significant main effect of day due to the increased success as training progressed ($F(13,546)=45.61$, $p<0.001$) and but no main effect of age ($F(1,42)=1.78$, $p=0.19$) or age x training day interaction ($F(13,546)=1.20$,

p=0.28). In the long duration training period, there was a significant age x training day interaction (F(44,616)=1.83, p=0.001), reflecting that the aged mice surpassed the young mice with ongoing training, but no main effect of age (F(1,14)=1.33, p=0.27).

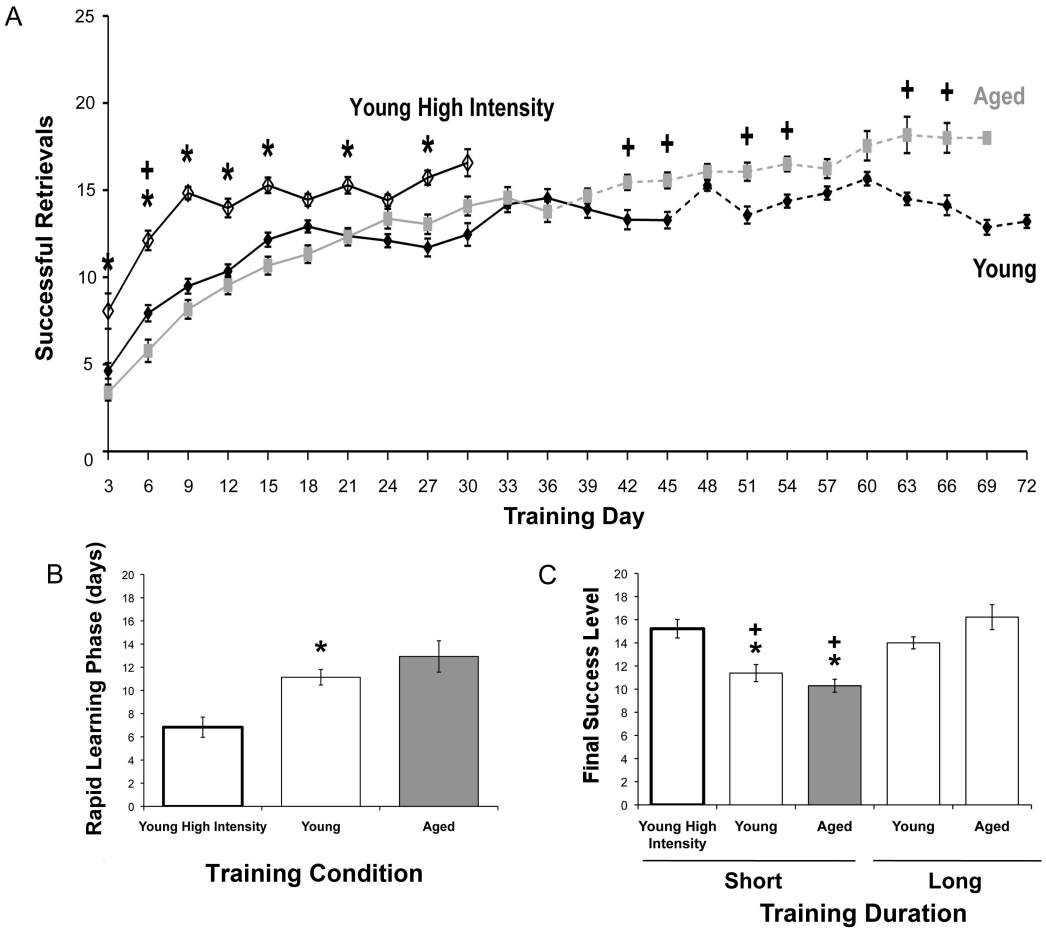


Figure 3.2 Young and aged mice learn and perform a skilled reaching task in a similar way. (A) Reaching success over 3 day training blocks in young and aged animals. The solid lines represent the combined averages of short and long duration trained animals. The dotted lines represent only the long duration trained animals. *p<0.05, young vs. young high intensity, +p<0.05, young vs. aged. (B) The average number of days until animals transitioned from a rapid learning phase to more gradual improvements. *p<0.05, compared to high intensity. (C) Final success level, measured as the average number of successful retrievals averaged over the last 3 training days. Data are the same as presented in A, but presented here to highlight the differences in final success rates between the short and long duration groups. *p<0.05, compared to high intensity, +p<0.05, compared to long duration. All data are means ± S.E.M.

Within young mice in the short duration period, there was a significant effect of training intensity ($F(1,32)=7.18$, $p=0.01$) and a significant intensity x training day interaction ($F(13,416)=2.01$, $p=0.02$). Young mice receiving high intensity training reached a near plateau in performance by the 9th training day, approximately 1 week earlier than young mice in the standard intensity training condition (Fig. 3.2A).

Both young and aged mice in the long duration training condition performed at a greater success rate at the end of the training period than age-matched animals in the short duration training condition ($F(4,49)=3.11$, $p=0.02$). These results show that increasing the intensity of training increased the task acquisition rate in young animals and aged animals were able to successfully learn and perform a skilled motor task.

3.4.1.2 Capellini Handling Test

Quantitative measures of reaching performance can be insensitive to differences in movement style. The Capellini Handling Test, a test of coordinated dexterous forepaw use during pasta eating, allows measurement of skillful adjustments of the forepaws and atypical eating patterns. When all types of forepaw adjustments were considered together, aged mice had a greater number of adjustments during pasta handling than young mice ($t(13)=-5.12$, $p<0.001$). Though a greater number of adjustments can indicate greater dexterity (Allred et al., 2008), further analysis showed that aged mice make more adjustments that do not result in the advancement of the pasta piece into the mouth ("superfluous adjustments", $t(13)=-5.80$, $p<0.001$; Tennant et al., 2010b). However, even when these superfluous adjustments are subtracted from the total number of adjustments,

aged mice still made more adjustments when compared to young mice ($t(13)=-2.81$, $p=0.02$). Aged mice also took significantly longer to consume each pasta piece ($t(13)=-8.35$, $p<0.001$; Fig. 3.3B) and to make each adjustment ($t(13)=-2.24$, $p=0.04$; Fig. 3.3C).

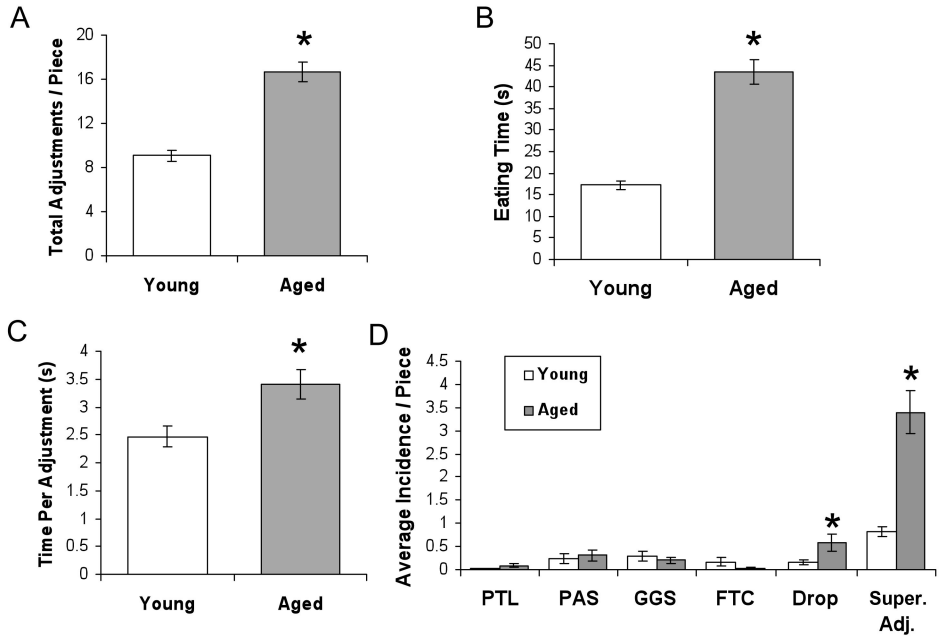


Figure 3.3 Behavioral performance on the Capellini Handling Test. (A) Average number of adjustments per paw per pasta piece. (B) Average time to eat each pasta piece. (C) Average time, in seconds, per adjustment, calculated by dividing the time to eat by the number of adjustments. (D) Average number of atypical behaviors, separated by category. PTL = paws together when long, PAS = paws apart when short, GGS = guide and grasp limb switch, FTC = failure to contact pasta with one limb, Drop = dropping the pasta piece during eating, Super. Adj. = superfluous adjustments do not result in advancement of the pasta into the mouth. *p<0.05. All data are means ± S.E.M.

Analysis of atypical handling behaviors (Fig. 3.3D) showed that aged mice drop the pasta piece during eating significantly more often than young mice ($t(13)=-1.65$, $p=0.03$). (Drop time is not counted in total eating time.) However, aged and young mice did not

differ significantly in the incidence of other atypical behaviors, with the exception of superfluous adjustments, as discussed above.

3.4.1.3 Ladder Rung Walking Test

On the Ladder Rung Walking Test, a test of coordinated paw use during locomotion, aged mice had a significant slowing in the time taken to cross a horizontally aligned ladder ($t(13)=-3.57$, $p=0.003$; Fig. 3.4A), and a nearly significant increase in the number of errors per step ($t(45)=-9.25$, $p=0.06$; Fig. 3.4B) compared with younger mice. Aged animals took more steps while crossing the ladder ($t(46)=-2.44$, $p=0.02$), but the mean step length of young mice (6.35 ± 0.06, measured in number of ladder rungs crossed) was only slightly longer than that of aged mice (5.73 ± 0.06; $t(13)=1.72$, $p=0.11$).

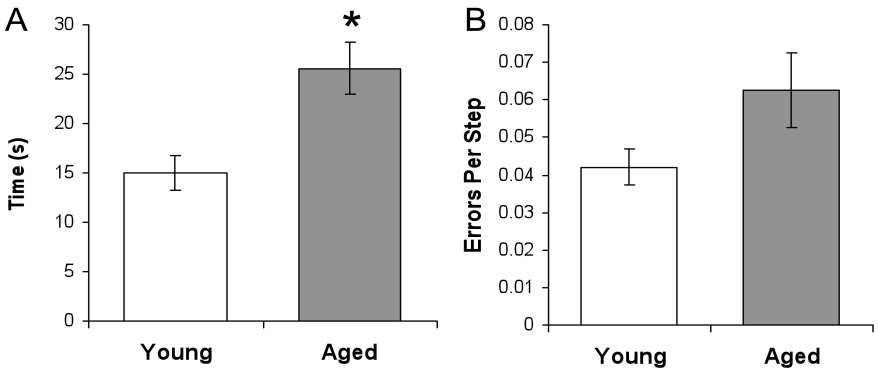


Figure 3.4 Behavioral performance of aged mice is slowed on the Ladder Rung Test. (A) Average time, in seconds, to cross the full extent of the ladder. (B) The average number of errors (footslips) divided by the total number of steps. *p=0.003. Data are means ± S.E.M.

3.4.1.4 Bilateral Tactile Stimulation Test

In the Bilateral Tactile Stimulation Test, small pieces of tape are placed on the ventral surface of both paws to be removed by the mouse. Aged mice showed a significant delay in the time taken to contact either stimulus ($t(478)=-3.57$, $p=0.0003$; Fig. 3.5A). Aged mice differences in the amount of time to completely remove the stimuli did not reach significance ($t(476)=-1.72$, $p=0.09$; Fig 3.5B).

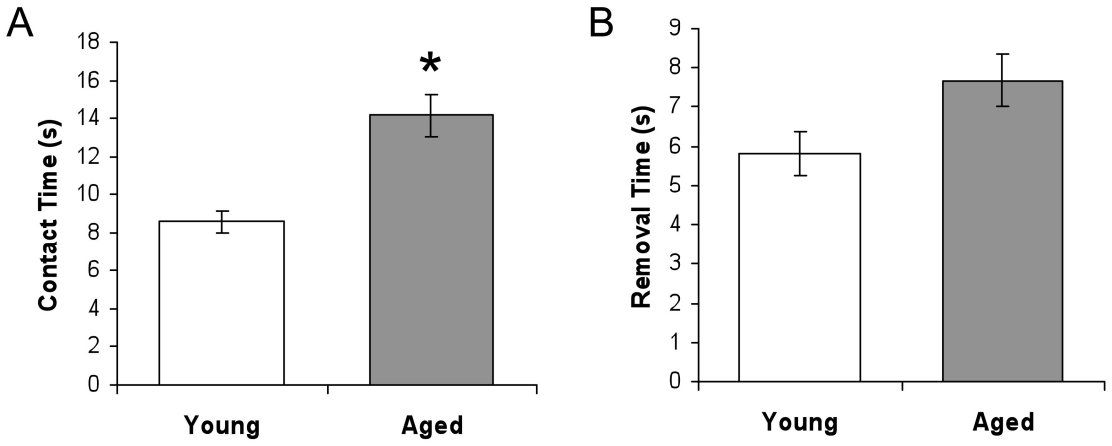


Figure 3.5 Aged mice take longer to contact stimuli during the Bilateral Tactile Stimulation Test. *(A)* Average time to first contact each stimulus. *(B)* Average removal time for each stimulus, calculated by subtracting the contact time from the total time to remove each stimulus. *$p=0.0004$. All data are means ± S.E.M.

3.4.2 Effect of age on the organization of the forelimb area of motor cortex

The forelimb motor map of young adult mice is composed of a large caudal forelimb area (CFA) and a smaller rostral forelimb area (RFA), similar to that of the rat. Young adult mice differ from rats in having a large relative area of digit representations in the CFA (Chapter 2; Tennant et al., 2011). Inclusive of mice in all training conditions, aged mice had a significantly smaller area of the CFA composed of digit representation

($t(124)=2.93$, $p=0.004$) and a significantly larger area composed of wrist representation compared to young mice ($t(124)=-2.66$, $p=0.009$, Fig. 3.6A). There were no differences in the elbow representation area or the total areal extent of the CFA in young and aged animals (1.45 ± 0.05 vs. 1.51 ± 0.09 mm^2, respectively). The ratio of proximal (elbow) to distal (digit and wrist) forelimb representation in the CFA was similar across ages (young: 0.61 ± 0.06 mm^2; aged: 0.59 ± 0.07 mm^2). Additionally, in long duration trained animals, in which complete shoulder representations were mapped, there was no significant difference in the area of shoulder representations between young and aged mice (0.38 ± 0.05 vs. 0.31 ± 0.06 mm^2, respectively)

The percentage of animals with a discernable RFA was similar between age groups (73 % vs. 69 %, aged vs. young; $\chi^2(1, N = 109) = 0.20$, $p = 0.35$). Among mice with an evident RFA, the RFA of aged animals was significantly larger in area than that of young animals (24 ± 2 vs.18 ± 1 mm^2, respectively; $t(87)=-2.53$, $p=0.01$). The larger RFA in aged mice consisted mainly of an increase in the wrist representation area, which resulted in a significantly larger area of distal forelimb representations compared to proximal representations (Fig 3.6B).

3.4.3 Effect of reach training on the forelimb motor maps of young and aged mice

Previous research has shown that, following skill training, the overall map area can remain unchanged while there is internal reorganization of the movement subtypes (e.g., enlargement of distal at the expense of proximal forelimb representations; Kleim et al. 1998). Increases in representation area of one movement type typically accompanies a

decrease in areas of one or more other movement types. In rats, areas that are smallest at baseline (i.e. distal forelimb representations) increase at the expense of the areas that are largest at baseline (i.e. proximal forelimb representations; Kleim et al., 1998). As a result, ratios of proximal to distal forelimb representations (or vice versa) have been useful for quantifying reorganization of movement representations during motor learning (Kleim et al., 1998). Because mice typically have smaller elbow representations at baseline (Chapter 2; Tennant et al., 2011) and based on preliminary results, we hypothesized that the elbow representation would expand at the expense of distal representations.

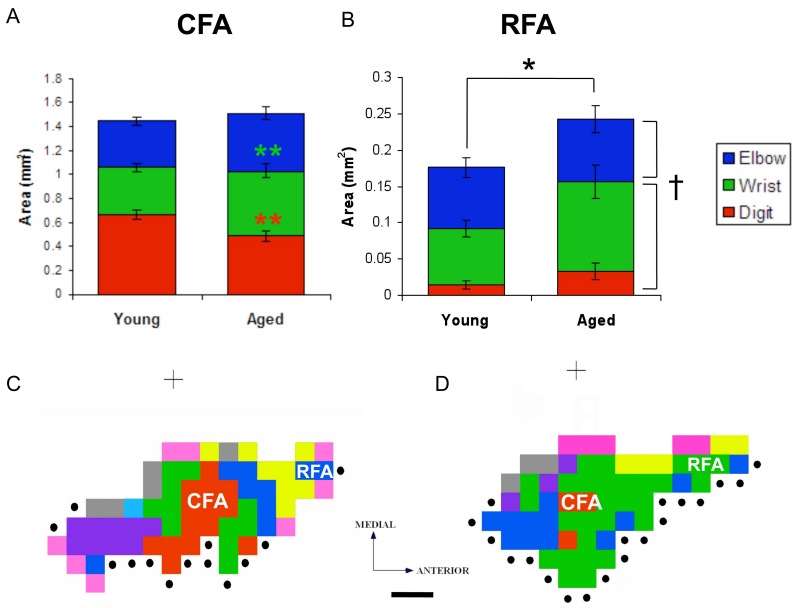


Figure 3.6 Aging results in a decrease in the digit representations in the caudal forelimb area (CFA) and an enlargement of the rostral forelimb area (RFA). *(A)* Areal extents of elbow, wrist, and digit representations in young and aged animals in the CFA. *(B)* Areal extents of elbow, wrist, and digit representations in young and aged animals in the RFA. Brackets delineate proximal (elbow) and distal (wrist and digit) forelimb representations. *(C,D) R*epresentative maps from a young mouse *(C)* and an aged mouse *(D)*, indicating CFA and RFA. *p<0.05, **p<0.01, Young vs. Aged; †p<0.05, proximal vs. distal. All data are means ± S.E.M. Scale bar equals 500 µm.

There were no significant differences between training groups in the absolute areas of the individual or total forelimb representations in the CFA of young mice or aged mice (Table A1). Motor cortical maps examined after the shorter duration of training were found to have a significantly higher ratio of proximal to distal forelimb representations compared to controls ($F(1,22)=4.43$, $p=0.048$, short duration and high intensity vs. controls; $F(1,22)=4.59$, $p=0.04$, short duration vs. controls) but there was no effect of high intensity vs. low intensity training ($F(1,14)=0.99$, $p=0.34$). After a longer duration of training, proximal to distal ratios tended to decrease in comparison to those found after a short duration of training ($F(1,18)=3.99$, $p=0.06$, short duration vs. long duration) to levels that were not significantly different from controls ($F(1,18)=0.66$, $p=0.43$, controls vs. long duration). Even when shoulder was included in analysis of proximal forelimb representation area, the proximal to distal forelimb ratio of long duration reach trained animals was not significantly different from controls ($F(1,18)=1.80$, $p=0.20$). Animals in the delayed mapping condition, which underwent a 4 week period of rest between the end of training and ICMS mapping, showed ratios that were not significantly different from short duration trained animals ($F(1,17)=0.001$, $p=0.97$). However, the delayed mapping group had more variability in its proximal to distal ratios and they also were not significantly different from controls ($F(1,15)=0.94$, $p=0.35$). Pasta handling alone had no effect on the proximal to distal ratio compared to controls ($F(1,35)=0.65$, $p=0.43$).

In aged mice, short duration reach training had no significant effect on proximal to distal ratios ($F(1,15)=0.03$, $p=0.87$, short duration vs. controls) and ratios were similar

in mice with long and short durations of training (F(1,12)=0.34, p=0.57). Long duration reach trained animals were not significantly different from controls when the analysis included shoulder representations ($F(1,9)=0.11$, $p=0.75$) or when it did not ($F(1,9)=0.10$, $p=0.76$). Pasta Handling also had no effect on proximal to distal forelimb ratios compared to controls (F(1,27)=0.10, p=0.75).

Unlike CFA, in young adults, training tended to result in changes in RFA that varied with intensity (Fig. 3.8) but these effects failed to reach significance. When only short duration reach trained animals were compared to controls, there was a tendency of the lower intensity training to increase the size of the RFA compared to controls ($F(1,12)=4.44$; $p=0.06$, short duration vs. controls). This tendency was not seen with high intensity training, which tended to result in a smaller RFA than controls ($F(1,10)=3.46$, $p=0.10$, controls vs. high intensity). There was no effect of duration ($F(1,11)=0.01$, $p=0.93$, controls vs. long duration) or a delay prior to ICMS mapping ($F(1,11)=0.01$, $p=0.93$, controls vs. delayed maps). Additionally, pasta handling alone had no effect on the organization of the young RFA compared to controls ($F(1,24)=0.42$, $p=0.52$). Results in the aged RFA were similar to those seen in the young RFA (Fig. 3.8). There was no significant effect of short duration reach training ($F(1,9)=0.12$; $p=0.74$) or a longer duration of reach training ($F(1,5)=0.39$, $p=0.57$) compared to controls. There were no differences between training conditions in proportions of individual forelimb representations in the young or aged RFA (Table A3).

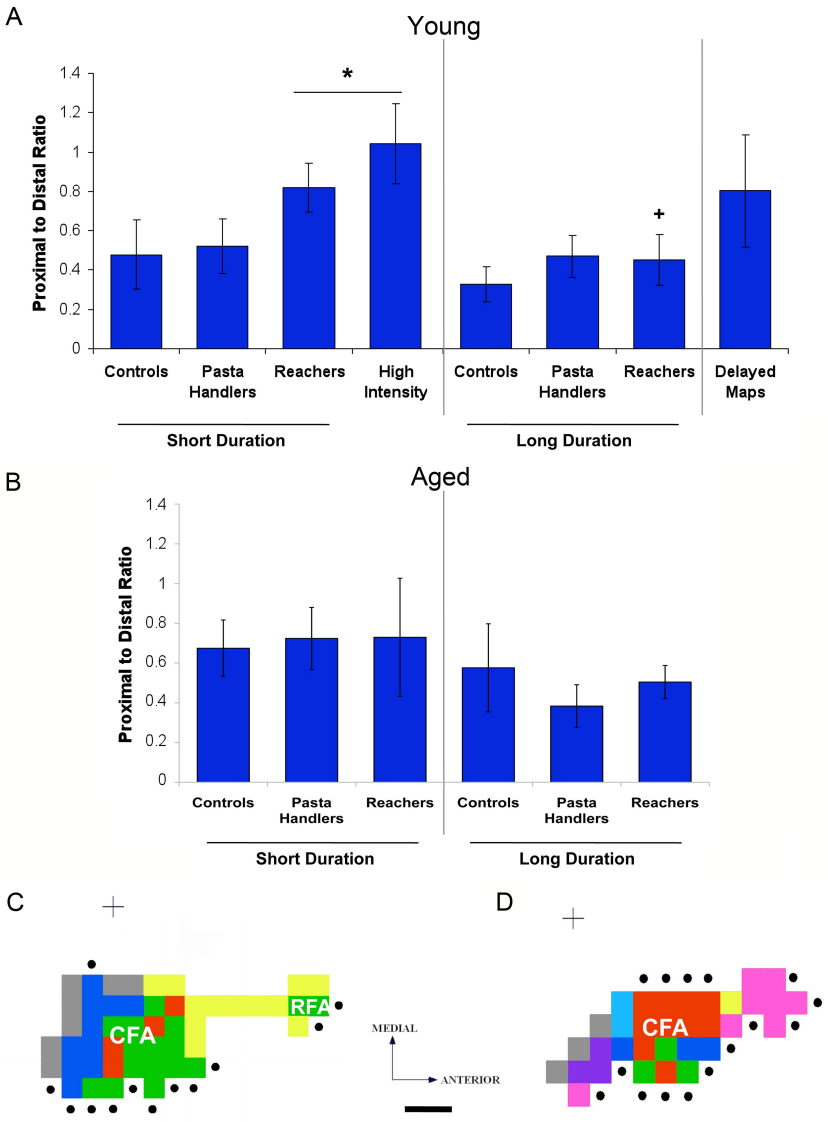


Figure 3.7 Training-induced plasticity occurs in the CFA in young mice following short duration reach training. (A,B) Ratios of proximal to distal forelimb representations within the CFA across animals with various levels and durations of behavioral experience in (A) young mice and (B) aged mice. (C) A representative map from a short duration reach trained animal. (D) A representative map from a long duration reach trained animal. This mouse had no discernable RFA. *$p<0.05$, young short duration reach trained animals (Reachers + High Intensity) compared to young controls. +$p<0.05$, young short duration reachers (Reachers + High Intensity) compared to young long duration reachers. All data are means ± S.E.M. Scale bar equals 500 μm.

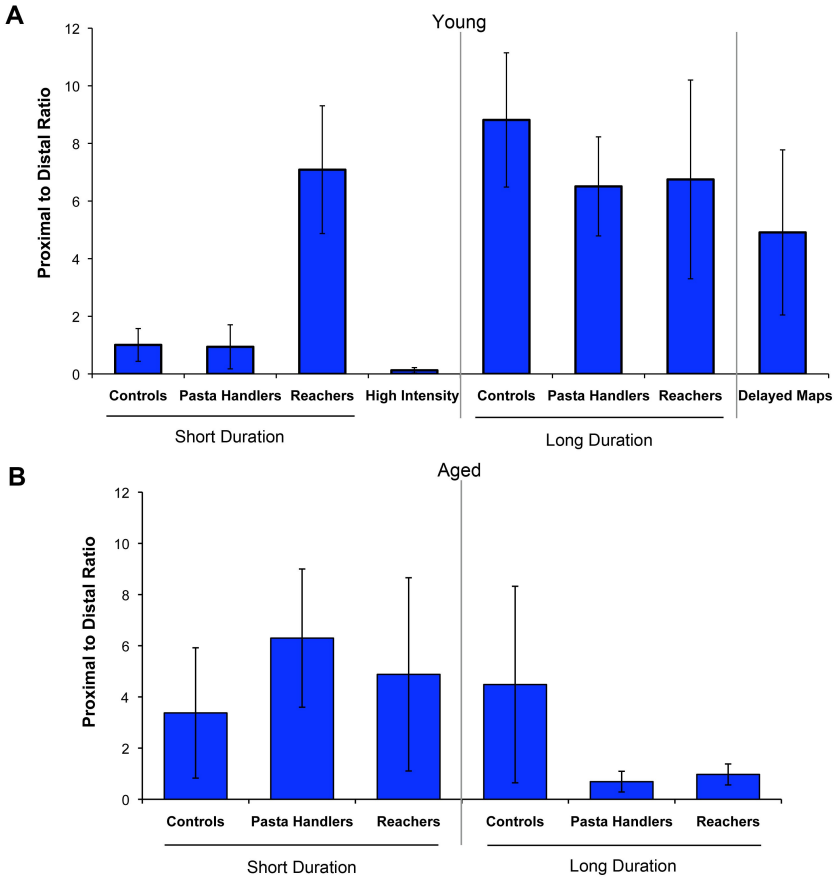


Figure 3.8 Ratios of proximal to distal forelimb movement representations in RFA of (A) young mice and (B) aged mice. Note the difference in scale relative to Fig. 3.7. All data are means ± S.E.M.

3.4.4 Movement thresholds

Although the reorganization of movement representations returned to control levels following a long duration of reach training in young mice, the movement thresholds of wrist and digit movements were lower than those of short duration reach trained mice (wrist: $t(15)=2.80$, $p=0.01$; digit: $t(12)=3.08$, $p=0.01$) and the delayed mapping group (wrist: $t(14)=-2.64$, $p=0.02$; digit: $t(15)=-2.63$, $p=0.02$; Fig. 3.9). In aged mice, there were no significant effects on movement thresholds of the elbow, wrist or

digit due to reach training (*F*s=0.04-2.81, *p*s=0.10-0.84), duration of reach training (*F*s=0.16-0.51, *p*s=0.49-0.70), or pasta handling (*F*s=0.05-0.52, *p*s=0.48-0.82). Mean thresholds were 29 ± 1 for elbow movements, 32 ± 1 for wrist movements and 38 ± 1 for digit movements.

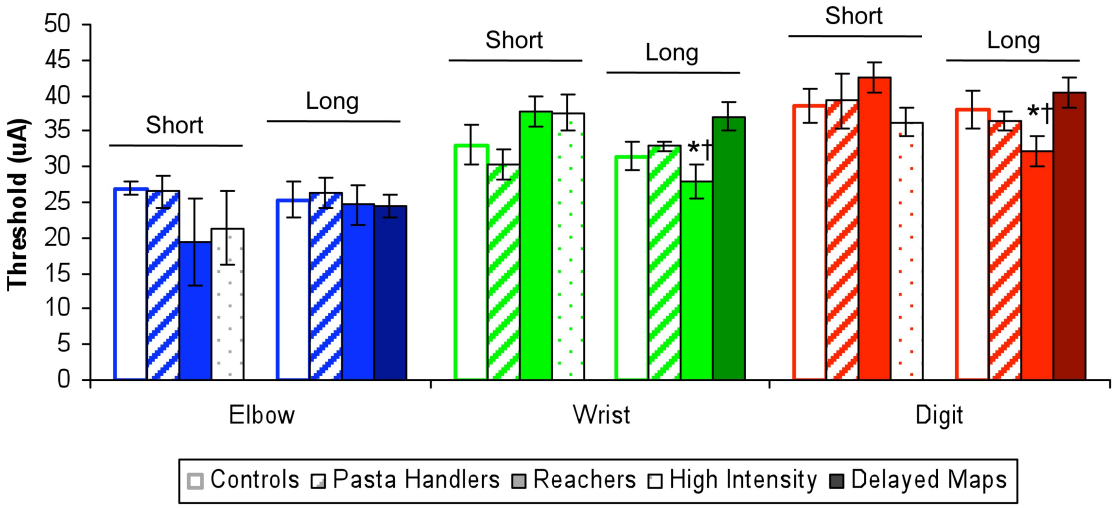


Figure 3.9 Distal forelimb movement thresholds decreased within the CFA of young animals following long duration reach training. *p<0.01, short vs. long duration Reachers, †p<0.05, long duration Reachers vs. Delayed Maps condition. All data are means ± S.E.M.

3.5 Discussion

Our results indicate that skilled reach training in young adult mice results in reorganization of the forelimb movement representations in the motor cortex, as evidenced by an increase in the ratio of proximal to distal forelimb representations. However, when the motor skill has been practiced for a long duration of time, the proximal to distal ratio returns to control levels. There are at least two possibilities for

why the increase in the proximal to distal ratio is transient. The first is that after a longer duration of training, the reorganized region of motor cortex is no longer required for performance of the task and the map returns to baseline (as found by Molina-Luna et al., 2008). However, this possibility conflicts with studies that have shown loss of motor skill following lesions of the sensorimotor cortex (Kleim et al., 2003; Adkins et al., 2004; Gharbawie et al., 2007) as well as findings of the long-term maintenance of new spines generated early in learning (Xu et al., 2009). A more likely possibility is that ICMS is revealing a transient stage in the long-term reorganization of the motor cortex that subserves the maintenance of the skill. Skilled learning-induced plasticity in the forelimb movement representation is thought to reflect the recruitment of neurons in the cortical territory devoted to producing novel movement sequences necessary for performing a newly learned motor skill (Keller, 1993). The interconnected nature of the motor representation is thought to provide the flexibility necessary to modify the existing network to accommodate this behavioral change (Sanes and Donoghue, 2000). In support of this, the representations of the digits, wrist, elbow and shoulder overlap within the forelimb representation area (Chapter 2; Tennant et al., 2011) as well as overlapping with the body representations that border the forelimb representations (Chapter 2; Tennant et al., 2011). The representation areas within the motor cortex are interconnected via layer II cortico-cortico connections, and plasticity of these connections is a proposed mechanism of motor map reorganization (Rioult-Pedotti et al., 2007).

It has been proposed that the process underlying reorganization of the motor map may be partially mediated by an LTP-like mechanism (Monfils and Teskey, 2004) and

that motor skill training results in a long-term increase in the synaptic modification range that allows for additional LTP in the motor cortex (Rioult-Pedotti et al., 2007). This potentiation, along with a transient net increase in spines seen during the early stages of motor skill learning (Xu et al., 2009), may result in the transient shift in movements most easily elicited by ICMS stimulation. There may be a prolonged time period in which the processes of synaptic pruning and potentiation are still ongoing and the resetting of the motor map may coincide with the full establishment of the circuitry underlying the ability to continue to perform the skill proficiently. In other words, the resetting of the forelimb motor map organization to control levels is unlikely to indicate a reversal of the plasticity, and it more likely indicates that the plastic changes are being more permanently incorporated into the motor cortical network, possibly through the addition of spines and/or synapses. For example, Kleim et al., (2002) showed that the number of synapses per neuron increased in the CFA, but not the RFA or hindlimb area, of rats that were trained on a skilled reaching task. Synaptogenesis was restricted to cortical motor representations that underwent reorganization. Additionally, although we found that the forelimb cortical motor map of mice returned to control levels after a longer duration of practice on the task, stimulation thresholds decreased for wrist and digit movements in this late time period. It is possible that lower movement thresholds are associated with increased synaptic efficacy (Monfils et al., 2005), and that the lower thresholds seen after extended training are due to an area-specific lasting synaptic potentiation that is maintained after the map has returned to control levels. A previous study by Molina-Luna et al. (2008) showed a transient enlargement of the rat motor cortex in response to motor

learning that returned to baseline after a period of no training. Despite the return to baseline, the ability to perform the skill was retained. Taken together, both this study and the current study suggest that motor map reorganization is necessary for acquisition of a skill, but that the maintenance of the reorganized state is not necessary for maintenance of the skill. Thus, motor map reorganization is transient whether or not the skill is continually practiced. However, it is possible that the stimulation parameters used by Molina-Luna et al., (2008) and by us in the current study were not sensitive to long-term changes in motor map organization. In rats, a different from of ICMS from the one used in this study, long-duration ICMS, produces complex, multi-joint movements which approximate reaching and grasping movements (Ramanathan et al., 2006). Reorganization of complex movement representations was seen following focal electrolytic lesions of the CFA and rehabilitative training, but not following short duration motor skill training in intact animals. It is possible that following long-term practice on a skilled motor task, the organization of complex movement representations may differ between animals trained for long and short durations.

Although the training-induced reorganization of the CFA in young animals was not fully maintained after the short duration period, movements of the wrist and digit became easier to induce. One theory is that lower movement thresholds are associated with increased synaptic efficacy (Monfils et al., 2005), and that the lower thresholds seen after extended training are due to plasticity at the synaptic level, which only occurs after the map has returned to control levels. The reduction in thresholds seems to be practice dependent because the thresholds of long duration Reachers are also significantly lower

than those of mice in the Delayed Maps condition (Fig 3.8). Thus, ongoing practice did not result in greater maintenance of maps compared to mice that ceased training, but decreased movement thresholds suggest that synaptic plasticity has also occurred and may last longer than motor map reorganization when a skill is continually practiced.

Aged animals were able to learn a skill in a similar time frame and with a similar success level as young mice. Towards the end of long duration training, the performance of aged mice even surpassed that of young mice. However, in contrast to young mice, we failed to see reorganization of the forelimb motor representations after short and long durations of training. In aged animals, the lack of reorganization within the motor cortical map agrees with evidence from the human literature showing dysfunction of the aged primary motor cortex (Siedler et al., 2010; Todd et al., 2010; Inuggi et al., 2011). In the human motor system, the aged brain is able to compensate for slowed processing capabilities by recruiting additional areas of the brain, including bilateral motor areas and ipsilateral sensory and cognitive areas, during performance of a motor task, allowing most aged individuals to perform at the same levels as their young counterparts during interlimb coordination tasks (Heuninckx et al., 2008). Tasks requiring a greater degree of dexterity, such as single digit or nondominant hand use, were associated with greater recruitment of additional brain areas (Hutchinson et al., 2002). However, even though older adults may be able to perform tasks at the same level as younger adults, their movements tend to be slower, especially on skilled tasks utilizing fine movements of the hand and digits, evidence that compensatory strategies may be in place to allow for better motor performance (Smith et al., 1999). Additionally, there is evidence that goal directed

reaching behavior leads to more successful reaching performance than repetitive gestures formed out of habit (Gholamrezaei and Whishaw, 2009). Anecdotal evidence from our studies suggests that aged mice remain more focused on the reaching task during daily training sessions than young mice, so the better performance of aged mice towards the end of long duration training may be due to an increased tendency for aged animals to have goal-directed reaching behavior while young animals develop habits that may increase success in the short term, but do not lead to long term task success. Overall, our results show that aging in mice has a similar effect on behavior and motor cortex function to that seen in humans. Additionally, given that motor learning in older humans involves more non-primary motor and non-motor areas compared to young adults, it could be that the motor cortex does not play as large a role in motor learning in aged animals as it does it young animals. Thus, stimulation-induced maps of primary motor cortex may not be the most sensitive method for detecting plasticity in the aged brain. Other brain areas that are candidates for motor skill learning-induced plasticity may include the somatosensory cortex, striatum, thalamus, internal capsule, cerebellum, and spinal cord. All of these areas play strong roles in motor learning or performance and more work needs to be done to determine what role each of these areas plays in motor skill performance in the young and aged brain.

The current study shows that training-induced motor map reorganization is transient in the young brain and that motor map plasticity is altered in aging. In healthy aged mice, the decline in motor function on some sensorimotor tasks was not reflected in the ability to learn a new motor skill. This suggests that motor skill learning and motor

performance must be at least somewhat dissociable. Our findings that training induced motor map reorganization in young animals dissipates with time agrees with the findings of Molina-Luna et al. (2008), but we have additionally found that ongoing practice on a task clearly does not result in maintenance of map reorganization. Given this result, and the differences in learning time courses of young and aged animals at the end of the long duration training period, it impossible to rule out that our failure to see reorganization of the aged motor map was due to mapping after the changes had already occurred, or that the reorganization was still to come. Remaining questions include the mechanism by which the "resetting" of the motor map occurs, whether learning an additional motor task will accelerate the "resetting" of the motor map and decreases in distal thresholds, and what effect increasing the intensity or lengthening the duration of training would have on the motor map of aged adults.

Chapter 4: Sensorimotor behavioral effects of endothelin-1 induced small cortical infarcts in C57BL/6 mice

4.1 Abstract

Mouse models have not paralleled rat models of stroke in advances in sensitive, species appropriate measures of neurological and behavioral recovery. Most available tests of mouse sensorimotor function are adaptations of those originally developed in rats and may not be as sensitive in detecting behavioral deficits after small cortical lesions in mice. Our purpose was to test the use of a vasoconstricting peptide, endothelin-1 (ET-1), to produce focal infarcts of the mouse sensorimotor cortex and to establish a behavioral test battery sensitive to resulting sensorimotor deficits. Young adult (3-5 month old) male C57BL/6 mice received intracortical infusions of ET-1 that produced unilateral lesions of the forelimb region of the sensorimotor cortex, intracortical infusions of sterile saline, or sham surgeries. Pre-operatively and at various time points over 3 weeks post-surgery, they were administered a test battery that included measures of sensorimotor asymmetry (Corner and Bilateral Tactile Stimulation Tests), coordinated forepaw use (Cylinder and Ladder Rung Tests), and dexterous forepaw function (Pasta Matrix Reaching Test). ET-1 infusions resulted in consistently placed, focal cortical infarcts and forelimb impairments as measured with the Ladder Rung, Bilateral Tactile Stimulation, and Pasta Matrix Reaching Tests. On the Bilateral Tactile Stimulation and Pasta Matrix Reaching Tests, impairments persisted throughout the time span of observation (26 days). These results

support ET-1 as a viable option for creating small, reproducible lesions of anatomical subregions in the mouse neocortex that result in lasting functional impairments in the forelimb, as observed with sufficiently sensitive measures.

* This chapter has been published as Tennant KA, Jones TA. 2009. Sensorimotor behavioral effects of endothelin-1 induced small cortical infarcts in C57BL/6 mice. J Neurosci Methods. 181(1):18-26.

4.2 Introduction

Due to the increased availability of transgenic lines of mice, and the applicability of many of these mouse models to stroke research, there has been a surge in interest in developing sensitive behavioral assays of recovery of function in mouse models of stroke (Branchi & Ricceri, 2002; Bućan & Abel, 2002; Zhang et al., 2002; Li et al., 2004; Farr et al., 2006; Bouët et al., 2007). However, many mouse studies still use lesion size as their sole outcome measure. Although lesion size often correlates with functional deficits (e.g. Grabowski et al., 1993; Peeling et al., 2001; Hsu & Jones, 2006), it is not a sufficient indicator of recovery. For example, Binkofski et al. (2001) found in human stroke survivors that the amount of spared motor function was a better predictor of eventual recovery than lesion size. It is therefore essential for their clinical applicability that animal models of stroke include functional assessments, as noted by many previous investigators (e.g. Zivin & Grotta, 1990; Hunter et al., 1995; Corbett & Nurse, 1998; Jones et al., 1999; Cenci et al., 2002; Kleim et al., 2007). Most of the behavioral measures currently used with mice are adapted from those used with rats and their effectiveness in detecting mouse deficits has not been well determined. Thus, there is a

need to more firmly establish sensitive behavioral test batteries for mouse models of stroke, including tests that detect lasting functional deficits.

There is also a need to develop different focal infarct models in mice. Most mouse stroke studies have induced lesions by occlusion of the middle cerebral artery (MCAo), which may not model many aspects of smaller, more survivable, strokes (Carmichael, 2005) and, for many basic research questions, are less desirable than lesions in well defined anatomical subregions (e.g., Nudo & Milliken, 1996; Nudo et al., 1996b; Conner et al., 2003; Kleim et al., 2003b). In rats, endothelin-1 (ET-1), a powerful vasoconstricting peptide, can be applied to the cortical surface (Fuxe et al., 1997; Adkins et al., 2004) or injected intracortically (Fuxe et al., 1992; Hughes et al., 2003; Gilmour et al., 2004) to produce small lesions that are generally restricted to desired regions of the cortex (Windle et al., 2006). The period of vasoconstriction can last up to 16 hours and is followed by a gradual reperfusion over the next 48 hours (Biernaskie et al., 2001) that resembles the reperfusion time-course that occurs after some human strokes (Domingo et al., 2000). ET-1 induced lesions of the forelimb area of the sensorimotor cortex have been shown to produce reaching deficits in rats similar to those produced by aspiration and excitotoxic lesions (Bury & Jones, 2002; Voorhies & Jones, 2002; Gilmour et al., 2004; Luke et al., 2004; O'Bryant et al., 2007) and behavioral deficits and neuroplastic changes in the contralesional hemisphere that resemble those produced by similarly placed electrolytic lesions (Adkins et al., 2004; Jones & Schallert, 1992).

ET-1 has also been used as an alternative to MCAo and photothrombosis to produce cerebral ischemic lesions in the mouse brain. Wang et al. (2007) found that

intracortical infusions of ET-1 into C57BL/6 mouse brains caused a 70-80% reduction in blood flow, followed by the formation of a small lesion that was restricted to cortex. Mice showed Rotorod and neurological score deficits when tested at 1 hour, but not 3 days, post-ischemia. Horie et al. (2008) found that neither cortical nor striatal infusions of ET-1 produced significant lesions in C57BL/6 mice unless combined with occlusion of the common carotid artery (CCAo) and co-administration of the nitric oxide synthase (NOS)-inhibitor, *N(G)*-nitro-L-arginine methyl ester (L-NAME). The mice given striatal infusions of ET-1 and L-NAME did show deficits in the Schallert Cylinder Test at 2 days post-infarct, and the addition of CCAo caused an even greater deficit. These somewhat ambiguous results suggest that there is a need for further characterization of the behavioral deficits that result when using the ET-1 model in mice.

The current study investigated sensorimotor impairments caused by intracortical infusions of ET-1 into the forelimb area of the mouse sensorimotor cortex. A battery of sensorimotor tests was used in an attempt to characterize the nature of resulting deficits over time after the lesion. This included measures of sensorimotor asymmetry, using the Corner (Zhang et al., 2002; Li et al., 2004; Bouët et al., 2007) and Bilateral Tactile Stimulation Tests (Schallert et al., 1982; Schallert & Whishaw, 1984; Starkey et al., 2005; Wells et al., 2005; Bouët et al., 2007). The Cylinder (Schallert et al., 2000; Baskin et al., 2003; Fleming et al., 2004; Li et al., 2004; Starkey et al., 2005; Wells et al., 2005) and Ladder Rung Tests (Metz & Whishaw, 2002; Riek-Burchardt et al., 2004; Farr et al., 2006) were used as measures of coordinated forepaw use. Finally, the Pasta Matrix Reaching Test, originally developed for rats (Ballermann et al., 2001; see also Teskey et

al., 2003; Chiken & Tokuno, 2005), was newly adapted for mice in the current study as a measure of dexterous forepaw function.

4.3 Materials and methods

4.3.1 Subjects

A total of 19 well-handled 3-5 month old male C57BL/6 mice were housed in groups of three to four with standardized housing supplementation (a small piece of PVC pipe, a cardboard roll, and small wooden objects) on a 12:12 light/dark cycle. Animals were maintained on a restricted feeding schedule (3 g/day) to prevent satiation and motivate reaching performance. Ten mice received intracortical infusions of ET-1 (Fig. 4.1A), three mice received intracortical infusions of 0.9% sterile saline, and six mice received sham procedures. One of the 10 mice given ET-1 lesions died during recovery from perioperative anesthesia and another failed to show evidence of a lesion in behavioral and histological analyses and was omitted from the study. An additional 13 animals used in a different study were used for limb length analysis. The animals were matched for age, sex, and strain. Reaching data from a subset of these animals (n=8) was used to determine the average number of days until asymptote and final success level on the Pasta Matrix Reaching Task. Animal use was in accordance with a protocol approved by the University of Texas at Austin Animal Care and Use Committee.

4.3.2 Intracortical infusion of ET-1

Following preoperative testing on the sensorimotor battery, mice were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). When fully anesthetized, as verified by tail/foot pinch and corneal response, the scalp was shaved and cleaned with providone-iodine. Each mouse was then placed into a mouse stereotaxic frame (Stoelting, Wood Dale, IL), lidocaine (2 mg/kg, s.c.) was injected into the scalp, and a midline incision was made. A small burr hole was drilled through the skull over the center of the forelimb region of the sensorimotor cortex at coordinates of 2.25 mm lateral to midline and + 0.6 mm anterior to Bregma. The dura was punctured and a Hamilton syringe with a 26 gauge needle was lowered into the cortex to a depth of 700 μm. Four μl of ET-1 (American Peptide; 320 pmol, 0.2 μg/μl in sterile saline) was injected into the cortex over the course of 10 min, and the syringe was left in place for 5 min following infusion to prevent backflow. In rats, topical application of ET-1 results in little horizontal spread beyond the craniectomy borders (Adkins et al., 2004). The burr hole was then filled with gelfoam and covered with UV curing dental cement (wave A2; Southern Dental Industries, Victoria, Australia), and the wound was sutured and covered in antibiotic ointment. The animal was allowed to fully awaken in a heated chamber before returning to its home cage. Of the two sham groups, one group received all surgical procedures up to the skull opening and the other received a skull opening and infusion of vehicle (0.9% sterile saline) into the forelimb area of the sensorimotor cortex.

4.3.3 Behavioral Methods

In order to acclimate mice to the behavioral testing procedures, they were exposed to the behavioral apparati at least once during the 2 weeks prior to the initiation of training. For the Pasta Matrix Test, mice first received pieces of capellini pasta in their home cages over several days to reduce neophobic responses. Mice were then trained daily on the Pasta Matrix Test for 15 days before surgery. They were tested on the remainder of the sensorimotor battery once per week for two weeks prior to surgery, and on days 2, 4, 10, and 20 post-surgery. Daily post-operative training on the Pasta Matrix Test was initiated 4 days after surgery.

4.3.3.1 Pasta Matrix Reaching Test

This task involves training mice to reach for and break small pieces (3.2 cm in height and 1 mm diameter) of vertically oriented, uncooked capellini pasta (DeCecco brand, Fratelli De Cecco di Filippo Fara San Martino S.p.A., Italy), arranged in a matrix distal and lateral to the reaching chamber aperture (Fig. 4.2A). The animal must change its reach trajectory in order to obtain pasta pieces further from the reaching aperture. The methods used were adapted from rat versions developed by Ballermann et al. (2001) and used by Teskey et al. (2003) and Chiken & Tokuno (2005). The chamber was composed of four Plexiglas walls (20 cm tall, 15 cm long, and 8.5 cm wide) with an open top and bottom. The matrix was positioned in front of the reaching aperture, a center slit (13 cm tall and 5 mm wide) cut into the front wall of the chamber. The matrix itself was composed of a heavy-duty plastic block (8.5 cm long, 5 cm wide, and 1.5 cm tall) with 1

mm diameter holes drilled completely through. There were a total of 260 holes, beginning 2 mm from the reaching window with 2 mm between each hole. We expected the matrix size to exceed the maximum reaching distance of the mice.

In order to successfully retrieve a pasta piece, the mouse must break the pasta by grasping and pulling forward. The matrix is designed so that the pasta piece extends through the entire depth of the matrix stage so that approximately half the piece is exposed. This prevents the mice from grasping and pulling the pieces out of the matrix, and forces them to break the pieces in order to retrieve them. The movement sequence of the rat performing the Pasta Matrix Reaching Task was described by Ballermann et al. (2001) as a six step sequence: aim, digits open, pronation, grasp, withdrawal, and eat. Mice display approximately the same sequence of movements with the exception that they typically show little pronation of the paw before grasping the pasta. While rats grasp the pasta from the top, around the tip, mice grasp the pasta from the side, around the middle.

Mice were trained on the Pasta Matrix Reaching Test for 15 days before surgery to establish the skill and then were tested on it daily beginning 4 days after surgery. For pre-operative training, mice first underwent 3-5 days of shaping in order to become accustomed to the reaching task. During this time, the matrix stage was completely filled with pasta, allowing mice to reach for pasta with both limbs. Training began when mice reached at least 10 times in 15 min, and at least 70% of the time with one limb (termed the "preferred" limb). Two mice did not learn how to reach and were excluded from testing on the Pasta Matrix Reaching Task but were included in the remainder of the

sensorimotor battery. One of the two mice would not eat pasta (before lesion induction). Of the mice that learned how to reach, 9 had a left limb preference and 7 had a right limb preference. Lesion and sham-operation procedures were contralateral to the preferred limb. Mice were trained to reach with the preferred limb by filling with pasta only the half of the matrix contralateral to this limb. Daily training sessions consisted of up to 100 reaches or 15 min, whichever occurred first. The average number of reach attempts per session was 65.68 ± 1.38 and was not different between Lesion and Sham groups post-operatively. In order to encourage reaching and focus the mice, the experimenter held a piece of pasta perpendicularly oriented against the back of the most easily accessed piece of pasta in the matrix. The number of pasta pieces successfully broken was recorded, as was the area of the matrix that the mouse cleared of pasta. Data were pooled across three day blocks in order to simplify presentation.

4.3.3.2. Schallert Cylinder Test

This test measures the use of the forepaws for postural support behavior during exploratory movements. Mice were placed into a Plexiglas cylinder (12.7 cm in diameter, 25.4 cm tall) and allowed to vertically explore the cylinder walls while being videotaped with a digital camcorder (Canon XL1S). This test has been used previously in mice (Baskin et al., 2003; Fleming et al., 2004; Li et al., 2004; Starkey et al., 2005; Wells et al., 2005) and was adapted from the rat version of the test (Schallert et al., 2000). Mice were removed from the cylinder after completing 10 rears (approximately 5 min). Rears were counted to ensure that a sufficient number of behavioral observations could be

obtained from video playback (because, on average, each rear is followed by numerous instances of postural support with the paws against the cylinder wall). On average 39.4 ± 0.8 observations of forepaw use were recorded per test session using this method. Videotapes were analyzed in slow motion, and the number of times each paw was used to contact the cylinder wall and push off from or land on the floor were recorded for up to 30 total contacts. An asymmetry score was calculated for each movement type using the following formula (using contacts as an example): (# of ipsilesional contacts + 1/2 bilateral contacts) / (# of ipsilesional + contralesional + bilateral contacts) x 100.

4.3.3.3. Corner Test

This test is sensitive to responsiveness to somatosensory stimulation in mice (Zhang et al., 2002; Li et al., 2004; Bouët et al., 2007). Mice were allowed to walk into a corner formed by two Plexiglas boards, each 20 cm x 30 cm and fused into a 30 degree angle. As the mouse neared the corner, the two boards delivered bilateral vibrissae stimulation, and the mouse would rear up and turn out of the corner. The direction of rearing and turning was recorded for 10 trials. For a trial to be considered complete, the turn must have been preceded by a rear (Zhang et al., 2002). Trials in which the mouse turned without rearing were excluded from analysis. The percentage of ipsilesional turns was calculated as: (ipsilesional turns / 10) x 100.

4.3.3.4. Ladder Rung Test

Mice were videotaped while walking across a horizontal ladder (Fig. 4.4A). This test was originally developed for use with rats (Metz & Whishaw, 2002; Riek-Burchardt et al., 2004) and was later adapted for use with mice (Farr et al., 2006). The ladder rung apparatus was composed of an elevated horizontal ladder (80 cm long and 12 cm in elevation) with the home cage at the far end of the ladder. The rungs (121 in total) were 1 mm in diameter and evenly spaced 5 mm apart, and the ladder had Plexiglas sides (15 cm tall) to prevent the mouse from turning around or jumping off. The mouse was placed on the end of the ladder away from the home cage and videotaped while walking across the ladder rungs towards the home cage. Videotapes were scored using frame-by-frame analysis (Final Cut software) for step length (measured as the number of rungs crossed over) and qualitative score. Each step onto a ladder rung was qualitatively scored on a 3 point scale, abbreviated from that used by Farr et al. (2006): 1 = paw slips between or off a rung (Fig. 4.4B); 2 = paw is placed on a rung and readjusted on the same rung or placed onto a different rung; 3 = paw is well-placed onto the rung (Fig. 4.4A). The percent errors (slips), adjustments, and correct placements per step were calculated, along with average step length and average qualitative score.

4.3.3.5. Bilateral Tactile Stimulation Test

This test was adapted from those previously used with mice (Starkey et al., 2005; Wells et al., 2005; Bouët et al., 2007) and rats (Schallert et al., 1982; Schallert & Whishaw, 1984). Each mouse was placed into a shallow transparent plastic container (8.5

cm tall, 18 cm in diameter) with an open top and allowed to habituate for 1 min. The mouse was picked up and lightly restrained by the scruff while a 1.27 cm long piece of 3 mm wide tape (crepe art tape, Office Depot, Delray Beach, FL) was placed onto the ventral side of each paw (Fig. 4.5A). The mouse was then placed back into the container and allowed to remove each piece of tape using its teeth. The latency to contact and remove each piece of tape was recorded for 5 trials, allowing 30 seconds of rest between each trial. The percentage of trials in which the ipsilesional stimulus was contacted or removed first was calculated using the following formula: ([# of trials on which the ipsilesional stimulus is the first contacted + # of trials on which the ipsilesonal stimulus is the first removed] / 10) x 100. Average removal time for each stimulus was calculated using the following formula: latency to remove – latency to contact, averaged across five trials (Starkey et al., 2005).

4.3.4. Histological euthanasia and tissue processing

Mice were euthanized with an overdose of sodium pentobarbital (175 mg/kg, i.p.) and perfused intracardially with 0.1 M phosphate buffer (PB) and 4 % paraformaldehyde. After perfusion, the length of the forearms (digit tip to elbow) was measured in a subset of mice (n=13) to relate to reaching distances in the Pasta Matrix Reaching test. Brains were stored in 4 % paraformaldehyde and sliced into 50 μm thick sections using a vibratome. Every sixth section was mounted onto gelatin-coated slides and Nissl stained with toluidine blue.

4.3.5. Analysis of remaining cortical volume

Neurolucida software was used to estimate the volume of remaining cortex. Coronal sections were viewed at a magnification of X51. The cortical areas of 6 coronal sections from approximately 2.0 mm anterior to 0.5 mm posterior to Bregma, each 300 μm apart, were measured by tracing their cortical boundaries. The sensorimotor cortex fell within the area of tissue measured, and no lesions extended outside of this area. Cavalieri's method was used to calculate total remaining cortical volume by multiplying the sum of the section areas by the distance between sections (Henery & Mayhew, 1989; Mayhew, 1992). Lesion volume was indirectly calculated by subtracting the volume of the damaged hemisphere from the volume of the intact hemisphere.

4.3.6. Statistical analyses

SPSS software was used to conduct repeated-measures analyses of variance (ANOVAs) for all behavioral measures, with day as a within-subjects variable and group as a between-subjects variable. A t-test was conducted to compare the lesion extents of the Sham and ET-1 groups. The two Sham groups did not significantly differ from one another and were combined for all statistical analyses, except for the Corner Test analyses (see results). Following histological analysis, one animal in the ET-1 group was excluded from all analyses due to lack of an evident lesion. This animal also failed to show any behavioral deficits. An α level of 0.05 was considered significant for all analyses.

4.4 Results

4.4.1 Analysis of lesion extent

ET-1 induced lesions (n=9) resulted in damage to the sensorimotor cortex in 8 out of 9 mice. ET-1, which was injected 700 μm below the cortical surface, caused a pattern of damage that extended approximately 1 mm from anterior to posterior, and approximately 1 mm from medial to lateral surrounding the infusion site (Fig. 4.1A). None of the lesions produced damage to the underlying white matter or striatum. There

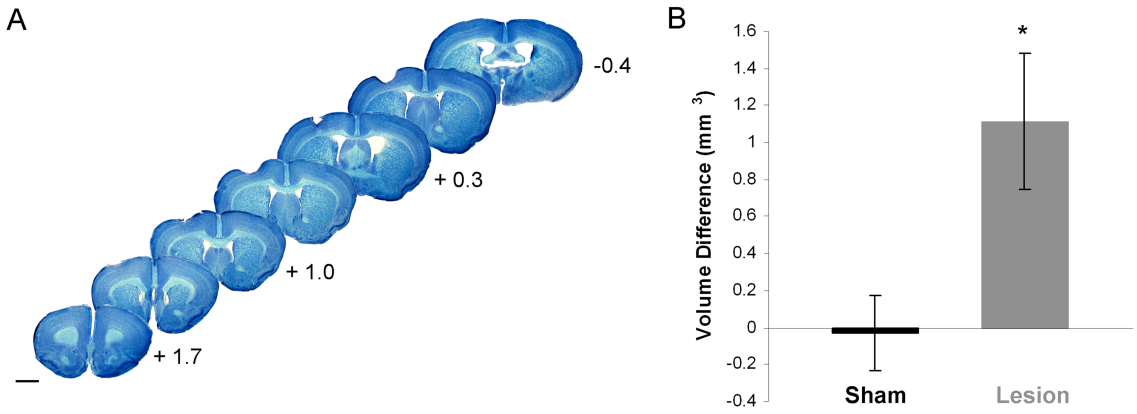


Figure 4.1 (A) Representative Nissl stained coronal sections of an endothelin-1 (ET-1) induced sensorimotor cortex lesion. Brain tissue was collected 26 days after lesion induction. Numbers to the right indicate approximate distances from Bregma in mm. Scale bar = 1 mm. (B) ET-1 lesions of the sensorimotor cortex resulted in a significant loss of sensorimotor cortex in the ipsilesional hemisphere as measured by the interhemispheric volume difference. *p< 0.01 significantly different from Sham.

was some damage to layer I of the cortex within the perilesion area (~250 µm in distance around the perimeter of the lesion), but there is no visible evidence of damage to the superficial layers of the cortex beyond this area, nor is there evidence of damage in the contralateral hemisphere. There was no difference in the volumes of the intact cortex of

Lesion and Sham animals (14.8 ± 0.5 and 14.7 ± 0.6 mm, respectively). Sham (saline) infusion procedures (n=3) resulted in minor damage associated with the cannula tract that was evident in coronal sections. As shown in Figure 4.1B, ET-1 (n=8) significantly increased the measured volume difference between the intact hemisphere and the ischemic hemisphere, as compared to sham operates (n=9) ($t(15)=-3.03$, $p=0.004$). There was no significant difference in interhemispheric volume between the Sham subgroups that received saline infusions versus no infusions.

4.4.2 Pasta Matrix Reaching Test

Mice became proficient on the Pasta Matrix Reaching Test during 15 days of pre-lesion training (Fig. 4.2B). Mice with ET-1 induced lesions (n=7) showed a deficit in skilled reaching on the Pasta Matrix Reaching Test as compared to sham operates (n=8) (Fig. 4.2C). There was a significant effect of day ($F(21,252)=2.81$, $p=0.00007$) and a significant day x group interaction ($F(21, 252)=1.96$, $p=0.008$). There was no significant main effect of group (Lesion versus Sham, $F(1,12)=2.02$, $p=0.18$). Post-hoc analyses indicated that the number of pasta pieces broken by the Lesion group was significantly different from the number broken by the Sham group during the first two weeks of post-operative training but was not significantly different from Sham levels in the final week of training. The Lesion group's post-operative performance was also significantly different from pre-operative performance during the first half of post-operative training and returned to pre-operative performance levels during the second half of training. Thus,

after 21 days of training, mice with ET-1 induced lesions seemed to show some behavioral recovery of reaching skill.

Not only did lesions cause a decrease in the number of pasta pieces broken, but the pattern of breakage was altered after lesion induction (Fig 4.3A). Prior to lesion induction, the majority of mice were able to reach pasta pieces located far anterior and far lateral to the reaching aperture. Mice were able to reach pieces as far as 1.2 cm (6 pieces) away from the reaching aperture. This corresponded well to the average length of the mouse forelimb that can be extended beyond the reaching aperture (1.96 ± 0.03 cm from elbow to digit tips). Early after lesion induction, the extent to which mice were able to successfully retrieve pasta pieces from the anterior and, especially, the lateral area of the matrix was limited. With time, most of the mice broadened the area of the matrix cleared. Examples of the areas cleared by a single mouse over the course of pre-lesion training and post-lesion testing are presented in Fig. 4.3B.

Performance of the Pasta Matrix Reaching Task can be also be measured by calculating the percentage of breaks per total reach attempts (percent success). The pattern of behavioral results following lesions was the same when calculated as % success because the average number of attempts on each day is not altered post-operatively. The behavioral deficit in the first two weeks after the ET-1 injection is not due to a generally reduced use of the affected limb because there is no decrease in the number of reach attempts made by the affected limb after lesion induction. In the number of reach attempts made per session, there was no significant change in pre- versus post-operative time points and no significant difference between sham and lesion groups.

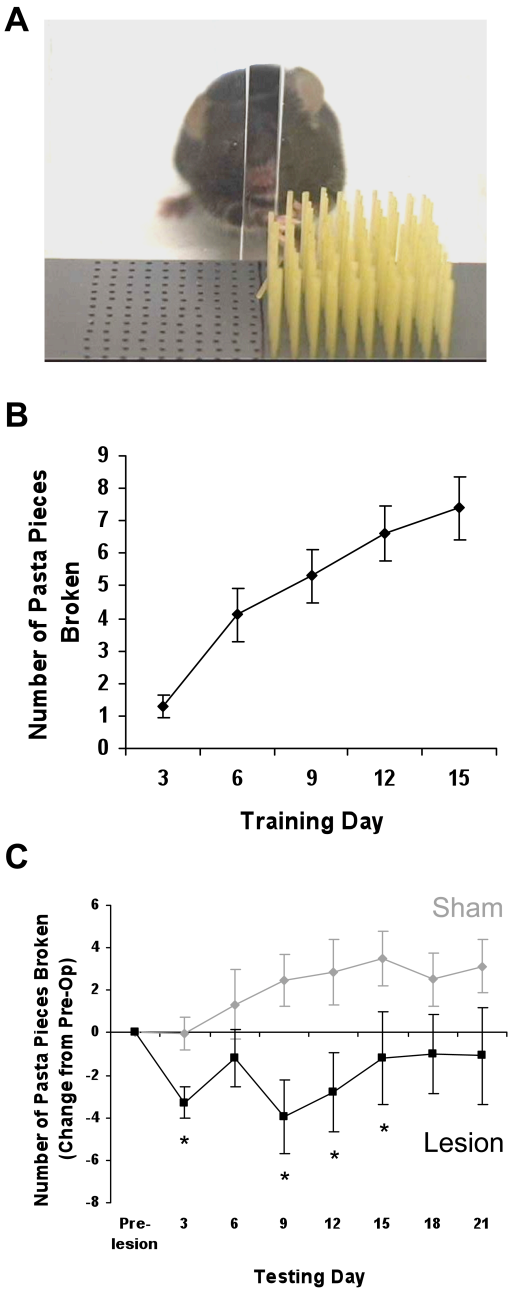


Figure 4.2 (A) A mouse performing the Pasta Matrix Reaching Test. The mouse reaches for pasta arranged in the side of the matrix contralateral to the trained reaching limb. (B) Mice received 15 days of pre-lesion training during which time they became proficient in performing the test. (C) The lesion resulted in significant impairments compared to the Sham group. Impairments were most evident in the first two weeks of post-lesion testing. Data are means ± SEM change from pre-operative performance (post-operative - pre-operative number of pieces). *p< 0.05 significantly different from Sham.

Pre-operatively, sham operates made 69.2 ± 11.0 attempts and the lesion group made 75.7 ± 12.5 attempts. Post-operatively (averaged over time points) sham-operates made 62.9 ± 7.6 and lesion groups made 72.1 ± 9.5 attempts.

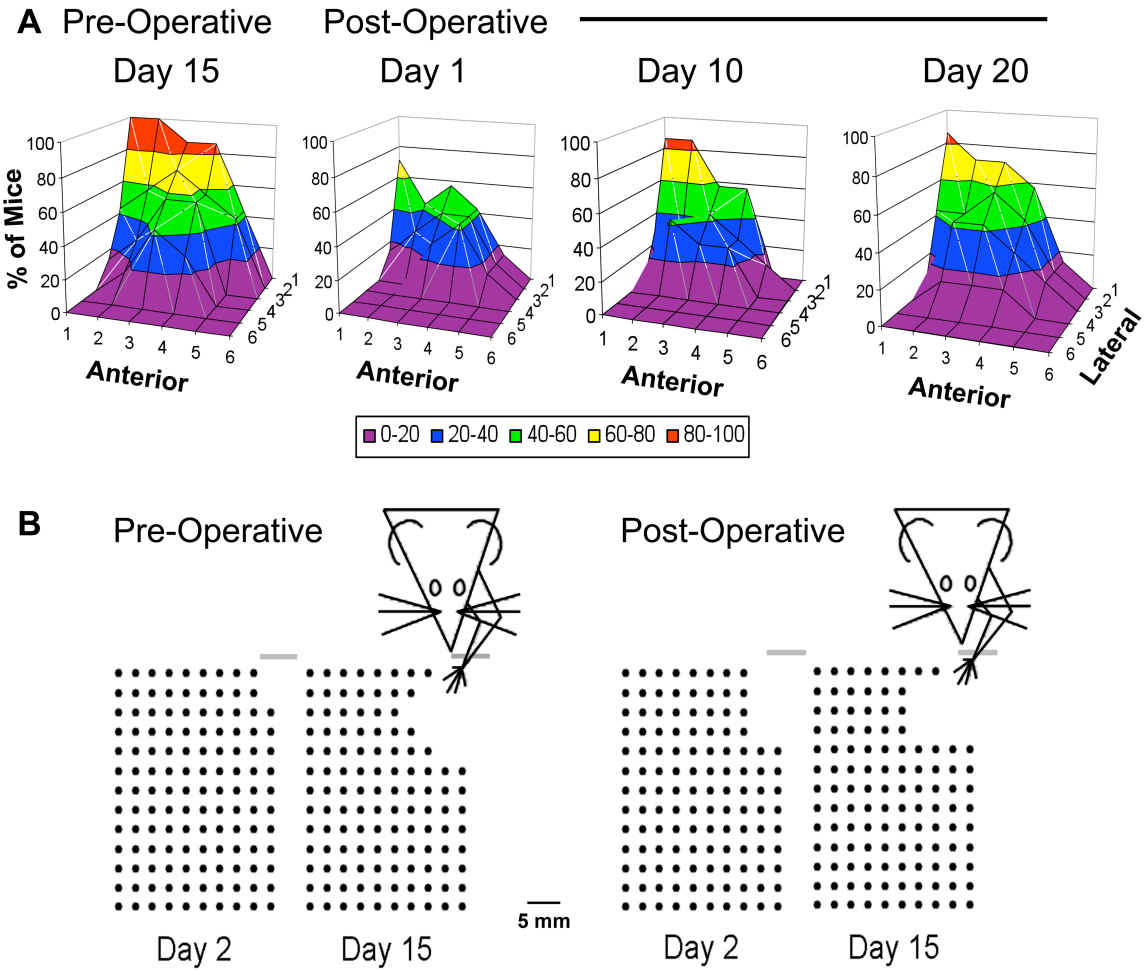


Figure 4.3 (A) Three-dimensional surface plots reflecting the percentage of animals in the Lesion group (n=7) that successfully broke a pasta piece at each location in the matrix on a given day. Horizontal axes represent the anterior (column) and posterior (row) number of the matrix, with anterior 1, lateral 1 being closest to the reaching aperture. (B) An example from a single mouse of the area of the matrix cleared over the course of initial learning of the task (pre-operative) and post-operative performance. The position of the reaching aperature in relation to the pasta-containing portion of the matrix is indicated by a gray bar. The mouse reaches through this aperture for pasta pieces placed contralateral to the reaching limb. Scale bar = 5 mm.

4.4.3 Ladder Rung Test

Prior to lesion induction, 3.60 ± 0.91% of total steps made by the Lesion group and 3.25 ± 0.61% of total steps made by the Sham group resulted in an error on the Ladder Rung Test. Two days after ET-1 induced lesions, mice showed an increase in the number of errors (Fig. 4.4B). However, this recovered quickly. When mice were tested at

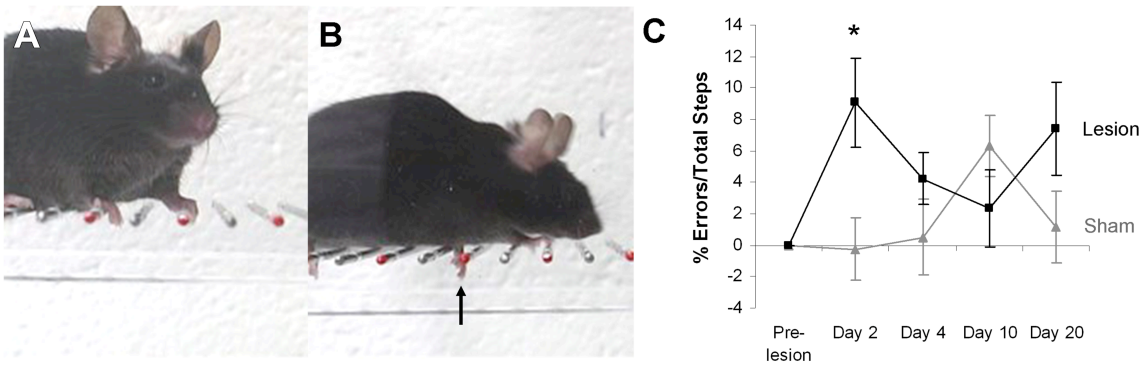


Figure 4.4 (A) A mouse performing the Ladder Rung Test. Both paws are placed correctly on the ladder rungs. (B) Example of an error on this test. The arrow indicates the limb that has slipped through the ladder rungs. (C) Lesions caused a transient increase in errors of the contralesional forelimb. Data are means ± SEM change from pre-operative performance (post-operative - pre-operative % errors/step). *p< 0.05 significantly different from Sham.

later time points, they no longer showed an increase in the number of errors made with the contralesional paw compared with the preoperative performance. There was a significant effect of day (F(4,27)=5.87, p=0.002) and a significant day x group interaction (F(4,27)=4.95, p=0.004), but no main effect of group. Post-hoc analyses indicated that on post-operative day 2 contralesional errors were significantly increased relative to Shams (Fig. 4.4C) and relative to the pre-operative performance level (p = 0.019). Errors with the ipsilesional paw were not significantly increased after the lesion

compared with the pre-operative time point or compared with Shams. On the other measures of performance on this test there were no significantly differences between Sham and Lesion groups, or between pre-operative and post-operative performance. Averaged over groups and days, the step distance (measured in number of rungs crossed), percent correct placements and percent adjustments per step were 5.13 ± 0.06, 82.62 ± 0.59%, and 11.24 ± 0.47 %, respectively. The average qualitative score was 2.73 ± 0.01.

4.4.4 Bilateral Tactile Stimulation Test

ET-1 induced lesions resulted in an asymmetry in responsiveness to tactile stimulation applied to the forelimb (Fig. 4.5B) as well as in a slight increase in time to remove the contralesional stimulus (Fig. 4.5C) on the Bilateral Tactile Stimulation Test. These changes are likely to reflect sensory and motor deficits, respectively, as discussed below.

The Sham group tended to respond equally to either stimulus, while the Lesion group consistently responded to the stimulus on the ipsilesional paw before the stimulus on the contralesional paw at all post-operative timepoints. There was a significant effect of group ($F(1,15)=5.27$, $p=0.037$), but no effect of day ($F(4,60)=1.76$, $p=0.15$) or day x group interaction ($F(4,60)= 1.31$, $p=0.28$) in response asymmetry.

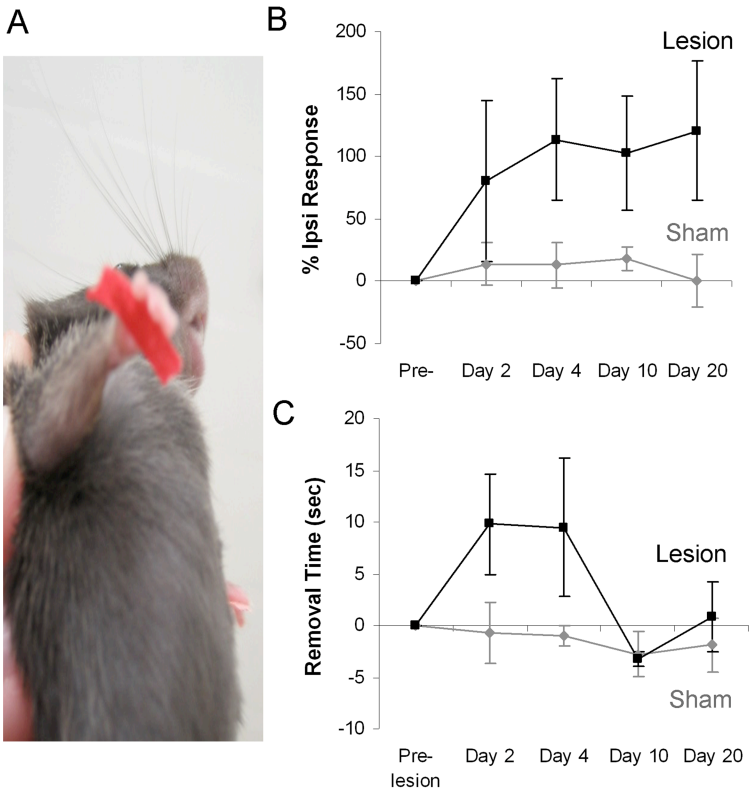


Figure 4.5 (A) Placement of the tape on the ventrum of the paw for the Bilateral Tactile Stimulation Test. Stimuli are placed on both paws for each trial and the order (left versus right) and latency for contact and removal are recorded. (B) Response bias. After the lesions, mice responded to the ipsilesional stimulus before responding to the contralesional stimulus on a greater percentage of trials compared to sham operates. (C) Response latency. Lesions tended to increase the latency to remove a stimulus from the contralesional forepaw, but this failed to reach significance (see text for details). Data are means ± SEM change from pre-operative performance of the contralesional limb (post-operative - pre-operative % ipsilateral, A, and time, B).

On days 2 and 4 post-lesion, the Lesion group took longer on average than Shams to remove the stimulus from the contralesional paw. However, when the Lesion and Sham groups were compared, the effects of group ($F(1,15)=4.39$, $p=0.053$), day ($F(4,60)=2.46$, $p=0.055$), and day x group interaction ($F(4,60)=1.69$, $p=0.16$) in the time to remove the contralateral stimulus all failed to reach significance in the removal time

measure. Prior to lesion induction, there were no significant differences between the Lesion and Sham groups in responsiveness to the ipsilateral stimulus (% ipsi first responses = 41.88 ± 8.55 and 59.44 ± 7.63, respectively) or time to remove the contralateral stimulus (7.80 ± 1.35 sec and 6.49 ± 1.95 sec, respectively).

4.4.5 Other behavioral measures

There were no significant lesion effects on either the Schallert Cylinder Test or the Corner Test (Table 4.1). The Lesion group showed no differences between pre- and post-operative performance (as measured on days 2, 4, 10, and 20 post-infarct with a one-way within-subjects analysis for time) in any of the Cylinder Test measures (contacts, push-offs, and landings, *F's*(4,28)=0.48-0.62, *p's*=0.75-0.65), or in performance on the Corner Test ($F(4,28)=0.59$, $p=0.67$). There were no significant Lesion versus Sham main effects or group x day interactions in the % use of the ipsilesional forepaw for any measure of the Schallert Cylinder Test (group by day: *F's*(4,60)=0.23-1.56, *p's*=0.92-0.20). Similarly, there was no significant group or group x day interaction in performance on the Corner Test (group by day: $F(4,60)=0.36$, $p=0.84$); however, this analysis was complicated by a post-operative difference between the two Sham groups. The Sham – Infusion group had a contralesional bias (turning ipsilesionally 26.7 ± 3.3 % of the time compared with 58.3 ± 7.0 % in the other Sham subgroup). The direction of the bias is opposite to that which would be predicted based on any injury in the saline-infused hemisphere (Zhang et al., 2002) and may reflect small sample size effects. Overall, the

pattern of results suggests that these two tests are insensitive to the effects of these lesions.

Table 4.1 Behavioral performance on the Cylinder and Corner Tests.

Test	Measure	Pre-Operative		Post-Operative	
		Sham	Lesion	Sham	Lesion
Cylinder	% Ipsi Contacts	54 ± 2	49 ± 2	51 ± 2	53 ± 2
	% Ipsi Pushoffs	42 ± 8	44 ± 6	40 ± 5	39 ± 9
	% Ipsi Landings	43 ± 4	47 ± 3	59 ± 4	50 ± 4
Corner	% Ipsi Turns	46 ± 6	51 ± 7	48 ± 7	63 ± 6

There were no significant differences in the performance of Sham and Lesion groups on the Cylinder and Corner Tests. Data are presented as the mean ± SEM performance at pre-operative and day 2 post-operative timepoints.

4.5 Discussion

In this study, we tested the suitability of ET-1 for producing focal infarcts in mouse cortex and also assessed the sensitivity of a mouse battery of behavioral tests for detecting sensorimotor impairments. Intracortical infusions of ET-1 into the forelimb area of the sensorimotor cortex caused small, focal lesions that were approximately 1 mm^3 in volume, centered around the infusion site and restricted to the cortex, causing no damage to the underlying white matter or striatum. The damage resulted in both acute and relatively long-lasting behavioral deficits on a subset of the tests of sensorimotor function.

The results support that intracortical infusions of ET-1 are a viable option for creating focal lesions of the mouse cortex for studies of cerebral ischemia. In addition to the ability to create anatomically defined lesions, a benefit of ET-1 stroke models is the gradual reperfusion that occurs, which may represent the time-course of some types of human stroke better than other reperfusion injury models (Domingo et al., 2000). However, this lesion method is not without its limitations. The ET-1 model differs from most human strokes in that it involves vasoconstriction rather than occlusion of vasculature. ET-1 is known to have direct effects on neurons and glia (reviewed in Rubanyi & Polokoff, 1994). These effects cannot be said to account for the damage caused by ET-1 application because co-administration with vasodilators prevents the injury (Fuxe et al., 1992). However, they may influence later cellular responses to the injury. Finally, as used in this study, the lesion method requires a small craniotomy and disruption of the dura, which together can create behavioral deficits in the absence of a lesion (Adams et al., 1994), as well as damage due to the insertion of an infusion needle into the cortex. However, as measured in the present study, vehicle infusion did not result in sensorimotor impairments contralateral to the infusion or loss of cortical volume.

Although some behavioral testing has been done on mice following ET-1 lesions, we are the first to document behavioral deficits that persisted beyond 3 days post-infarct. The same concentration and volume of ET-1 that can create large lesions in the rat brain (Windle et al., 2006) creates smaller lesions in the mouse cortex (Wang et al., 2007; Horie et al., 2008). Wang et al. (2007) found that ET-1 created small lesions of the cortex that resulted in deficits in neurological and Rotorod scores at 1 hour post-infarct,

but not at 3 days post-infarct. Horie et al. (2008) found that only when ET-1 infusions were combined with the administration of L-NAME could a lesion be detected. These animals showed a significant impairment on the Cylinder Test at 2 days post-lesion, and additionally occluding the CCA resulted in an even greater deficit. Our findings show that ET-1 alone produces significant loss of tissue that results in clear behavioral deficits when measured with sufficiently sensitive tests of forelimb function. The present results also suggest that Horie et al.'s (2008) failure to find more lasting behavioral deficits may be because the test that they used (Cylinder Test) is not very sensitive to these small cortical infarcts in mice.

Mice with cortical lesions in the current study had relatively long-lasting impairments on the Pasta Matrix Reaching Test, which measures skilled motor function, in particular dexterous function of the paws and digits. In rats and non-human primates, reaching tasks have served as important tools for studying neural mechanisms of motor skill learning and motor rehabilitation (Nudo & Milliken, 1996; Nudo et al., 1996a,b; Kleim et al., 1998; Maldonado et al., 2008). Thus, in addition to providing a highly sensitive measure of impairments in skilled motor function after cortical lesions, this test may be useful for studies of neural plasticity in response to skilled motor training. After 13 days of daily post-lesion training, mice with sensorimotor cortex lesions tended to show some recovery of motor skill, making the Pasta Matrix Reaching Test a possible rehabilitative training task for mice after ischemic lesions of the forelimb area of sensorimotor cortex. However, because the current study did not include an untrained control group, there is no way to tell if this recovery was spontaneous or due to the motor

skill training, and this requires further investigation. Also, the design of the Pasta Matrix apparatus requires further refinement. Mice were only able to reach a maximum of 18 pasta pieces when the matrix was filled only on the side contralateral to the trained limb. Decreasing the distance between pasta pieces so that there are more pieces within the normal reaching distance of the mouse may further increase the sensitivity of the test. Also, the continued improvement of the Sham group following sham-operative procedures suggests that mice had not reached asymptotic performance prior to lesion induction. It is expected that the group differences would be retained on this task even after training to asymptote because of the deficits seen on other motor tasks that do not require any previous training (the Ladder Rung Task and the Bilateral Tactile Stimulation Test). However, it is possible that greater pre-injury training would further increase the sensitivity of the task to lesion-induced impairments. Schubring-Giese et al (2007) found that, when forelimb sensorimotor cortical infarcts affect a previously established skill in rats, the speed of "re-learning" the task is slowed compared with rats that did not possess the skill prior to the injury. Thus, for the purpose of maximizing the sensitivity of this measure, future studies will establish a pre-operative performance criterion of several days of training past asymptotic performance based on individual learning curves.

The group difference in response asymmetry on the Bilateral Tactile Stimulation Test likely reflects a sensory bias towards the ipsilesional limb (Schallert et al., 1982; Schallert & Whishaw, 1984). The Lesion group showed no evidence of recovery from the response asymmetry over the days of testing, indicating that this test is very sensitive to relatively long-lasting impairments in sensory function. Removal time probably mainly

reveals a motor bias, since only the time *after* the stimulus was first contacted was taken into consideration in determining the latency to remove (Bradbury et al., 2002; Starkey et al., 2005). The measures of removal time approached significance. It is possible that placing smaller pieces of tape on the paw would reveal a greater deficit, as smaller pieces are more difficult to detect (Schallert et al., 1983) and may require greater dexterity to remove (though they may also require greater dexterity on the part of the experimenter to place correctly).

In the current study, the Ladder Rung Test detected transient impairments in the contralesional forelimb. Mice no longer showed a significant increase in the number of errors made with the contralesional paw when tested beyond two days post-lesion. It is very likely that variations in the test would detect more persistent deficits. For example, we did not vary the spaces between the ladder rungs, a method that has been shown to more sensitively detect fore- and hindlimb deficits (Metz & Whishaw, 2002). Furthermore, performances on similar grid walking tests are known to be sensitive to behavioral compensation, whereby animals appear to recover lost function on the task but are actually largely relying on other types of body movement (Bury & Jones, 2002). Thus, it is possible that the quick "recovery" on this test reflects behavioral compensation.

The Schallert Cylinder Test and the Corner test did not reveal sensorimotor deficits following intracortical infusions of ET-1 into the forelimb area of the mouse sensorimotor cortex. Previous studies with mice have used these tests to detect deficits caused by MCAo (Zhang et al., 2002; Li et al., 2004; Bouët et al., 2007). Our lesions,

however, did not damage the striatum, as is commonly seen after MCAo, and these tests may be more sensitive to striatal damage than to damage restricted to the cortex. For instance, in rats, lesions that caused nigrostriatal damage produce chronic post-operative asymmetries on the Cylinder Test, whereas lesions restricted to the forelimb area of the sensorimotor cortex (SMC) show most severe impairments on this measure early after the lesion, returning to more symmetrical forelimb use during the chronic phase (Schallert et al., 2000). However, the Cylinder Test is sensitive to focal ET-1 induced lesions of the forelimb area of the SMC in rats (Adkins et al., 2004) and, therefore, we are surprised not to find a similar effect on this measure in mice. This could be because these lesions in mice simply didn't result in sufficient impairments in postural support behaviors with the contralesional forelimb. Alternatively, it could be that a more challenging way of testing is needed to reveal this asymmetry. It could also be due to a species difference in performance on the test. Postural support challenges during upright exploratory movements may be greater in rats due to their morphology and larger size, while mice might be better able to compensate for any postural support deficits.

Although apparently less potent in producing lesions in the mouse brain than in the rat brain, the intracortical infusion of ET-1 into the mouse sensorimotor cortex does produce a small cortical infarct that results in behavioral deficits on tests of skilled motor function and sensorimotor asymmetry. We have established a battery of sensorimotor tests that can be used to detect behavioral deficits following sensorimotor cortex damage in the mouse, including a newly adapted version of the Pasta Matrix test of skilled reaching. These tests can be used to further explore the differences between mouse and

rat models of cerebral ischemia, and aid in advancing the mouse as a model organism for the study of stroke.

Chapter 5: Rehabilitative training on a previously acquired skilled reaching task is effective in promoting behavioral improvement after sensorimotor cortical lesions in young and aged mice

5.1 Abstract

The incidence of stroke in adulthood increases with advancing age, and the prognosis of behavioral improvement is poor in older stroke survivors. The purpose of the current study was to determine differences in behavioral improvement and motor cortical plasticity of young and aged animals following focal ischemic lesions of the sensorimotor cortex and rehabilitative training. Young and aged male C57BL/6 mice were trained on the Pasta Matrix Reaching Task for 2 months prior to lesion induction in order to develop proficiency in skilled reaching. Ischemic lesions of the forelimb area of the sensorimotor cortex were induced with endothelin-1 (ET-1). After a short recovery period, mice were either given no rehabilitative training, training on a novel reaching task, the Tray Reaching Task, or training on a previously learned task, the Pasta Matrix Reaching Task. Rehabilitative training continued for 9 weeks, and performance was probed once weekly on the Pasta Matrix Reaching test. Following the final probe session, mice underwent a terminal intracortical microstimulation (ICMS) procedure to resolve the extent of the forelimb motor representation in peri-lesion cortex. Pasta Matrix Reach training was effective in inducing behavioral improvement in both young and aged animals. Young animals showed some improvement after Tray Reaching, although not to the same extent as with Pasta Matrix training. Behavioral improvement in young mice was associated with an increase in the absolute area of the rostral forelimb area (RFA),

but we failed to see reorganization in the aged brain following 9 weeks of post-operative training. However, aged mice had significantly larger lesion volumes despite receiving a smaller volume of ET-1 during lesion induction. Thus, while our results indicate that rehabilitative training on a previously learned task after motor cortex lesions improves behavioral function in young and aged animals, reorganization of aged motor cortical representations may be stunted due to either age or lesion size effects.

5.2 Introduction

Stroke affects nearly 800,000 Americans each year (Roger et al., 2011). The large majority of strokes during adulthood occur over the age of 60, and the risk of stroke increases with advancing age. Adults over the age of 70 tend to be more disabled and dependent before stroke onset (Pohjasvaara et al., 1997; Kammersgaard et al., 2004; Rojas et al., 2007). Older age at stroke onset is associated with less gain in functional independence following physical therapy (Wang et al., 2011), and with greater likelihood of being discharged to a nursing home or private caregiver (Falconer et al., 1994).

Aged animal models of stroke are important for determining the efficacy of rehabilitative therapies for promoting behavioral improvements and beneficial neural plasticity. Following experimental induction of stroke, aged animals show long-lasting performance deficits on sensorimotor tasks such as ladder rung walking, bilateral tactile stimulation, and cylinder tests (Soleman et al., 2010). Rehabilitative tasks, such as reach training, have been shown to be effective in promoting behavioral improvements in middle-aged and aged rats and monkeys (Maldonado et al. 2008; Alaverdashvili and

Whishaw, 2010: Moore et al., 2010). Reaching success increases as both young and aged animals develop compensatory strategies for reaching. However, while young rats and monkeys' grasp patterns begin to normalize over time, older rats (Alaverdashvili and Whishaw, 2010) and monkeys' do not (Moore et al., 2000).

In the young adult squirrel monkey brain, motor cortical infarcts cause a reduction in the areal extent of the functional map and a loss of skilled forelimb use (Nudo and Milliken, 1996). Rehabilitative training on a reaching task induces a beneficial reorganization of the motor map concurrent with behavioral improvement (Nudo et al., 1996b). However, it is unknown what effect either stroke or rehabilitative training have on the aged motor cortical representations of the forelimb, which have already lost some complexity and plasticity in the intact brain (Chapter 3). The goal of the current study was to determine how aging affects the ability to regain a previously learned motor skill following focal ischemic motor cortical lesions and rehabilitative training on a previously learned or novel reaching task.

5.3 Methods

5.3.1 Subjects

A total of 25 male C57BL/6 mice aged 3-7 months and 24 male C57BL/6 mice aged 16-20 months were used. All mice were obtained from Jackson Laboratories (Bar Harbor, ME) at either 1 month of age or 9 months of age. Aged mice were either obtained as retired breeders (n = 17) or aged from 1 month in our colony (n = 17). After arriving in our colony, all animals were housed in groups of 3-4 except for retired breeders, who

were housed singly prior to arrival in our colony to prevent aggressive behavior. These differences in housing and histories were considered in the data analysis as explained below. All mice received standard cage supplementation, including a PVC tube, wooden toys to chew, cardboard tubes and paper nesting material. Following task acquisition and lesion induction, young and aged mice were randomly divided into three rehabilitative training conditions: No Rehabilitation (No Rehab; n=8 young, n=8 aged), Tray Reaching rehabilitation (Tray Rehab; n=8 young, n=8 aged), and Pasta Matrix Rehabilitation (PM Rehab; n=9 young, n=8 aged). Seven young and 10 aged mice were removed from the study due to post-operative mortality. These animals were not included in the animal counts above.

5.3.2 Endothelin-1 induced lesion surgeries

Following acquisition of the Pasta Matrix Reaching Task and pre-operative testing on the sensorimotor battery, mice were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). The scalp was shaved and cleaned with povidone-iodine, and the mouse was placed into a mouse-specific stereotaxic frame (Stoelting, Wood Dale, IL), lidocaine (2 mg/kg, s.c.) was injected into the scalp, and a midline incision was made. A small burr hole was drilled through the skull over the approximate center of the forelimb cortical motor representation at coordinates of 1.5 mm lateral to midline and +0.3 mm anterior to Bregma (Tennant et al., 2011). The dura was punctured and a calibrated pipette with a tip diameter of ~50 μm was lowered into the cortex to a depth of 800 μm. For young mice, 3.5 μl of ET-1 (American Peptide; 320 pmol, 0.2 μg/μl in sterile saline)

was infused into the cortex over the course of 10 min, and the syringe was left in place for 5 min following infusion to prevent backflow. Aged mice received a smaller volume of ET-1 (3 µl) based on a pilot study that indicated that the larger volume (and dose) increased mortality rates, but the remainder of the surgical procedure was identical to that of young mice. The burrhole was then filled with gelfoam and covered with UV curing dental cement (Wave A2; Southern Dental Industries, Victoria, Australia), and the wound was sutured and covered in antibiotic ointment. Each animal was allowed to fully awaken in a heated chamber and a single injection of buprenorphine (3 ml/kg, s.c.) was given before it was returned to the home cage. Post-lesion mortality rates were slightly higher for aged animals (29 % vs. 21 % for young animals), but this difference was not significant ($\chi^2(1, N = 66) = 0.49, p = 0.52$).

5.3.3 Behavioral methods

In order to acclimate mice to the behavioral testing procedures, they were exposed to the behavioral apparati twice in the 2 weeks prior to training onset. To reduce neophobic responses during the Pasta Matrix and Tray Reaching Tasks, mice received small pieces of capellini pasta and millet seeds in their home cages over several days. Mice were then trained daily on the Pasta Matrix Reaching Task for 2 months before surgery to ensure that all animals mastered the task. All mice were tested on the sensorimotor battery once per week for 2 weeks prior to surgery, and on days 2, 4, 10, 20, 40 and 60 post-surgery. The first probe of post-operative reaching success on the Pasta Matrix Reaching Test was initiated 4 days after surgery. All mice were probed on the

Pasta Matrix Reaching Task once per week, following a 5 day period of daily rehabilitative training on either the Pasta Matrix Reaching Task or the Tray Reaching Task or control procedures. Rehabilitative training and probe trials were continued in this fashion for 9 weeks total.

5.3.3.1 Pasta Matrix Reaching Task

This task involves training mice to reach for and break small pieces of vertically oriented, uncooked capellini pasta arranged in a matrix distal and lateral to the reaching chamber aperture (see Chapters 3 and 4 for detailed methods). In order to successfully retrieve a pasta piece, the mouse must break the pasta by grasping and pulling forward.

Pre-operatively, mice first underwent a 3–5 day shaping period in order to become accustomed to the reaching task. During this time, the matrix stage was completely filled with pasta, allowing mice to reach and grasp with either limb. Training began when mice reached at least 10 times in 15 minutes and showed a clear limb preference, defined as making at least 70 % of attempts with one limb. Mice were then trained to reach only with the preferred limb by filling only the half of the matrix contralateral to that limb (Figure 4.2). Daily training sessions consisted of up to 100 reach attempts or 15 minutes, whichever occurred first. The number of pasta pieces successfully broken was recorded, as was the area of the matrix that the mouse cleared of pasta. In the groups receiving rehabilitative training on the Pasta Matrix Reaching Task, post-operative training sessions were conducted exactly as pre-operative sessions. Mice that received Tray Reaching

rehabilitative training were also preoperatively trained on the Pasta Matrix Reaching Task, but were only exposed to the task again post-operatively during weekly probe trials.

In order to determine if age and ischemia affected the manner in which mice reached (Ballermann et al., 2001), weekly probe trials were videotaped. Frame-by-frame video analysis was used to analyze the following movement components: aim, advance, digits open, grasp, break, and withdraw. A movement was judged to be typical if it was similar to that seen in an animal prior to ischemia. The frequency of atypical movements in 3 successful reaches was recorded for each mouse prior to lesion induction and for each post-operative probe trial.

5.3.3.2 Tray Reaching Task

Of the groups receiving rehabilitative training, some received training on the Pasta Matrix Reaching Task, a task acquired pre-operatively, and others received training on the Tray Reaching Task, a task that they had no pre-operative experience with. Daily rehabilitation trials on the Tray Reaching Task consisted of a 15 minute session during which mice were allowed to reach for 100 millet seeds placed in an inclined glass tray (2.5 cm tall x 2.5 cm wide x 7.5 cm long) with a front lip (6 mm tall) to prevent scraping the seeds into the reaching chamber or retrieving seeds with the tongue (Fig 5.1). As mice cleared the tray in front of the window, the remaining seeds were periodically shifted towards the window by the experimenter in order to allow the mice to reach as many seeds as possible. Training was restricted to the contralesional limb by aligning the edge of the tray with the lateral edge of the reaching slit, on the mouse's contralesional side.

Mice were only able to successfully obtain seeds by reaching with the limb closest to the wall as the bulk of the seeds were contralateral to the reaching limb in that position.

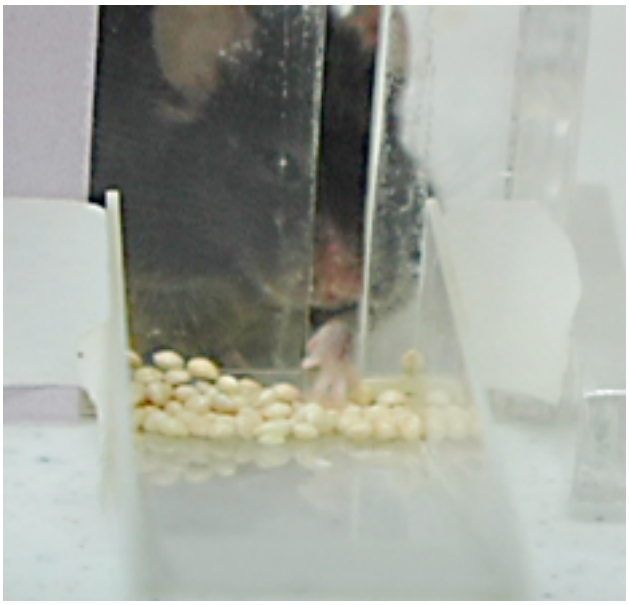


Figure 5.1 A mouse performing the Tray Reaching Task.

5.3.3.3 Capellini Handling Test

Mice were videotaped while eating short lengths (2.5 cm) of dried capellini pasta in a clear Plexiglas cylinder. A front-faced mirror was placed directly beneath the mouse in order to view paw movements because of the typical hunched posture of mice during pasta eating. The pasta was marked at evenly spaced intervals (0.8 cm) to facilitate video analysis. Trials consisted of 3-4 pieces of pasta per mouse. Trials were omitted if the paws became impossible to see (i.e., because the animal turned away from the camera). Slow motion video analysis was used to determine the number of adjustments made with each paw and the number of atypical eating behaviors per piece (described in Allred et al., 2008 and Tennant et al., 2010b). Data were averaged over three trials for each animal.

5.3.3.4 Ladder Rung Walking Test

Mice were videotaped while walking across a horizontal ladder composed of 121 1-mm diameter rungs 5 mm apart over a total distance of 80 cm (Fig. 4.4; Farr et al., 2006; Tennant and Jones, 2009). Plexiglas sides (15 cm tall) created a walkway for the animals. The ladder was placed onto a flat surface so that the rungs were elevated 1 cm above the tabletop so that mice had a surface to land on during larger footfaults. Providing a surface for the paw to land on prevents some of the compensatory behaviors that occur when animals try to avoid making larger footfaults on a highly elevated ladder (Zhao et al., 2005). Videotapes were scored using slow motion analysis for the total number of steps and footfaults with each paw and the total time to cross the ladder.

5.3.3.5 Bilateral Tactile Stimulation Test

Mice were placed into a shallow plastic container (8.5 cm tall, 18 cm in diameter) with an open top and allowed to habituate for 1 min. The mouse was then picked up and lightly restrained by the scruff while a 1.25 cm long piece of 3 mm wide tape (crepe art tape, Office Depot, Delray Beach, FL) was placed onto the ventral side of each paw (Fig. 4.5; Starkey et al., 2005; Wells et al., 2005; Bouët et al., 2007; Tennant and Jones, 2009). The mouse was placed back into the container and allowed to contact and remove each piece of tape using its teeth. The latency to contact and remove each piece of tape was recorded for three trials, allowing 30 s of rest between each trial. The time to contact each stimulus was averaged over the three trials. Removal time for each paw was calculated by

subtracting the latency to contract from the latency to remove the stimulus, averaged across three trials.

5.3.4 Intracortical microstimulation (ICMS) mapping

Mice underwent a terminal ICMS procedure 1-2 days following the final Pasta Matrix probe session. Motor cortical representation areas were defined by the movements generated at the lowest stimulation thresholds, the approach tradtionally used to characterize motor cortical maps in primates, rats, and mice (e.g. Nudo et al., 1996a,b; Kleim et al., 1998; Tennant et al., 2011; Young et al., 2011). Detailed methods are described in Chapter 2. Briefly, animals were anesthetized with an initial cocktail of ketamine (150 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and placed into a mouse stereotaxic frame (Stoelting). The cisterna magna was punctured to reduce cerebrospinal fluid volume, and the skull and dura overlying the motor cortex were removed. The craniotomy was then filled with warm (37 °C) silicone oil.

Using a hydraulic micropositioner, intracortical penetrations with a glass microelectrode (20-25 μm tip diameter) with a platinum wire were made at a depth of 790-800 μm (corresponding to mid- to deep-layer V). Penetrations were made at 250 μm intersections. At each site, a 40 ms train of 13 200 μs monophasic cathodal pulses was delivered at 350 Hz from an electrically isolated, constant current stimulator (BAK Electronics) at a rate of 1 Hz. Stimulation was increased up to a maximum of 100 μA, or until a visible movement was evoked on the contralateral side of the body. If a movement was evoked at or below 100 μA, the threshold current was determined by gradually

decreasing the stimulation until the movement stopped. The lowest amount of stimulation required to evoke movement was considered to be the threshold current. If no movement was seen at 100 μA, the site was considered non-responsive.

Electrode penetrations were made in a systematic order to minimize the contribution of the mapping procedure to inter-animal variability. The first electrode penetration was made in the likely CFA or hindlimb area, posterior to the center of the lesion, and penetrations were made in 250 μm increments moving in an anterior direction until a non-responsive or non-forelimb movement was elicited. Penetrations were made in a systematic order throughout the cortex, bordering in all forelimb responsive sites with non-forelimb or non-responsive sites. Non-responsive cortical areas have been found surrounding the lesion core of squirrel monkeys during ICMS procedures (Nudo and Milliken, 1996). Thus, penetrations were made throughout the typical extent of the mouse forelimb area (Chapter 2, Tennant et al. 2010) to ensure that all forelimb responsive sites were detected. Areal extents of remaining movement representations were calculated by multiplying the number of points corresponding to a specific movement (e.g., elbow) by the area of a single grid square (0.0625 mm^2).

5.3.5 Histological euthanasia and tissue processing

Following the end of the ICMS procedure, mice were euthanized with an overdose of sodium pentobarbital (175 mg/kg, i.p.) and perfused intracardially with 0.1 M phosphate buffer and 4 % paraformaldehyde. Brains were stored in 4 % paraformaldehyde for 1 week before slicing on a vibratome into 50 μm thick sections.

Every sixth section was mounted onto gelatin-coated slides and Nissl stained with toluidine blue.

5.3.6 Remaining cortical volume analysis

Neurolucida software was used to estimate the volume of remaining cortex. Coronal sections were viewed at a magnification of 50x. The cortical areas of 9 coronal sections per animal from approximately 2.0 mm anterior to 1.2 mm posterior to Bregma were measured by tracing their cortical boundaries. The sensorimotor cortex fell within the area of tissue measured, and no lesions extended outside of this area. Cavalieri's method was used to calculate total remaining cortical volume by multiplying the sum of the section areas by the distance between sections (Henery and Mayhew, 1989; Mayhew, 1992). Lesion volume was indirectly calculated by subtracting the volume of the damaged hemisphere from the volume of the intact hemisphere.

5.3.7 Statistical analysis

SPSS software was used for all statistical analyses. A two-tailed t-test was used to compare interhemispheric volume differences between young and aged mice. Correlations were conducted to relate volume differences to post-operative behavior and remaining forelimb representation areas. Repeated-measures analyses of variance (ANOVAs) were conducted to determine if quantitative and qualitative reaching success on probe trials and change from post-operative performance was affected by 1.) rehabilitative training in general (PM Rehab and Tray Rehab vs. No Rehab) or 2.) a

specific type of rehabilitative training (No Rehab vs. Tray Rehab and No Rehab vs. PM Rehab). Separate analyses were conducted for young and aged animals. Two-tailed t-tests were conducted to determine post-hoc group differences and to compare young and aged performance on pre-operative, initial post-operative, and post-rehabilitative training probe trials. Repeated-measures ANOVAs were also used to determine age by testing day effects on performance on the Capellini Handling, Ladder Rung Walking, and Bilateral Tactile Stimulaton Tests. *A priori* planned comparisons were conducted to compare training condition effects on the area of remaining forelimb cortical representations and the ratio of proximal to distal representations within remaining CFA and RFA. Planned comparisons were designed to test whether the remaining areas or ratio of proximal to distal forelimb representations were 1.) affected by rehabilitative training (PM Rehab vs. No Rehab and Tray Rehab vs. No Rehab) and 2.) whether motor maps varied depending on the type of rehabilitative training (Tray Rehab vs. PM Rehab).

5.4 Results

5.4.1 Remaining cortical volume analysis

Aged mice had significantly larger lesions than young mice, despite reducing the volume of ET-1 administered to aged mice. Lesion volume was indirectly calculated by subtracting the volume of the damaged SMC from the volume of the intact SMC. Absolute areas are included in Table 5.1. There was no difference in the absolute volume of the contralesional hemisphere between young and aged mice ($t(47)=0.29$, $p=0.77$). However, the ipsilesional hemisphere was significantly smaller in aged mice compared to

young mice ($t(47)=-3.92$, $p<0.001$). Interhemispheric volume differences were greater for aged mice than for young mice ($t(47)=-2.87$, $p=0.006$; Fig 5.2). Representative coronal sections from young and aged animals are shown in Fig. 5.2B. The contralesional and ipsilesional SMC volumes measured in these specific brains are 23.10 and 22.02 mm^3, respectively, for the young mouse and 24.54 and 19.49 mm^3 for the aged mouse. Lesion volume was found to correlate with reaching success on the final probe session on the Pasta Matrix Reaching Task ($r=-0.37$, $p=0.01$). Correlations failed to reach significance between lesion volume and initial performance deficits on the Pasta Matrix Reaching Task ($r=-0.25$, $p=0.09$) and the change from pre-operative performance on the final Pasta Matrix probe session ($r=-0.24$, $p=0.10$).

Table 5.1 Absolute volumes (mm^3) of ipsilesional and contralesional sensorimotor cortex.

	Ipsilesional SMC	**Contralesional SMC**
Young Mice	21.54 ± 0.51	21.97 ± 0.20
Aged Mice	20.25 ± 0.42*	22.47 ± 0.27

All data are means ± SEM. *$p<0.001$, young vs. aged.

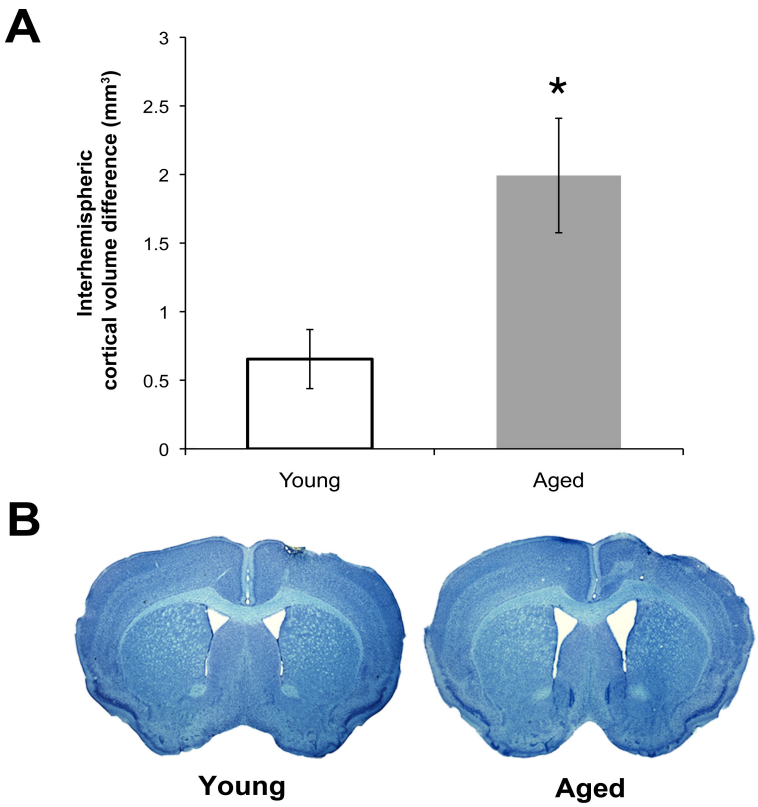


Figure 5.2 (A) Indirect measurements of lesion volume from young and aged mice following ET-1 induced focal ischemic lesions of the SMC. *p<0.01, Young vs. Aged. (B) Representative coronal sections from young and aged mice with ischemic lesions in the right hemisphere, showing the disparity in lesion extents.

5.4.2 Pasta Matrix Reaching probe trials

5.4.2.1 Young mice

Initially, all young mice had similar post-operative deficits in reaching. Tray Reaching and Pasta Matrix Reaching began to improve success levels in the first week of rehabilitative training. Mice had more successful post-operative reaching performance on the Pasta Matrix Reaching Task (which had been mastered prior to lesion) when they

received rehabilitative training on this task or a novel reaching task compared to mice that did not recieve rehabilitative training (Fig. 5.3A). When the results of weekly probe trials were compared across training conditions, we found that Pasta Matrix training resulted in a significant group effect ($F(1,15)=4.13$, $p=0.04$) but no day x group interaction ($F(10,150)=1.35$, $p=0.16$, PM Rehab vs. No Rehab). Tray Reaching training also resulted in a significant group effect ($F(1,14)=4.70$, $p=0.048$) but no day x group interaction ($F(10,140)=1.19$, $p=0.31$, Tray Rehab vs. No Rehab). Additionally, a comparison between the two rehabilitative training conditions showed that the two types of rehabilitative training produced similar behavioral improvements. There was no significant group effect ($F(1,15)=1.01$, $p=0.33$) nor day x group interaction ($F(10,150)=1.40$, $p=0.18$, Tray Rehab vs. PM Rehab). These results indicate that both Pasta Matrix and Tray Reaching were effective rehabilitative training tasks in young mice.

Video analysis of reaching movement components indicated that both Pasta Matrix and Tray Reaching training resulted in fewer atypical movements made by young mice. Tray Reaching training resulted in significant group effects in all reaching movements (Fs=6.47-16.70, ps=0.006-0.04), with the exception of grasping ($F(1,6)=0.41$, $p=0.54$), but no significant day x group interactions when compared to untrained controls. However, there was a nearly significant day x group interaction for breaking the pasta piece ($F(10,60)=1.98$, $p=0.052$). In comparison to controls, Pasta Matrix training resulted in significant group effects in all reaching movement

components (Fs=14.08-73.89, ps<0.001-0.007), with the exception of grasping ($F(1,7)=3.35$, $p=0.11$), and day x group interactions for advancing, opening the digits, and breaking the pasta (Fs=2.38-3.50, ps=0.001-0.02). A comparison between the two rehabilitative training conditions showed that the two types of rehabilitative training produced similar behavioral improvements. There was a day x group interaction for advancing ($F(10,70)=2.70$, $p=0.007$, Tray Rehab vs. PM Rehab), but no significant group effects. Thus, both Tray Reaching and Pasta Matrix Reaching effectively improved the manner in which mice reached for pasta to a similar extent.

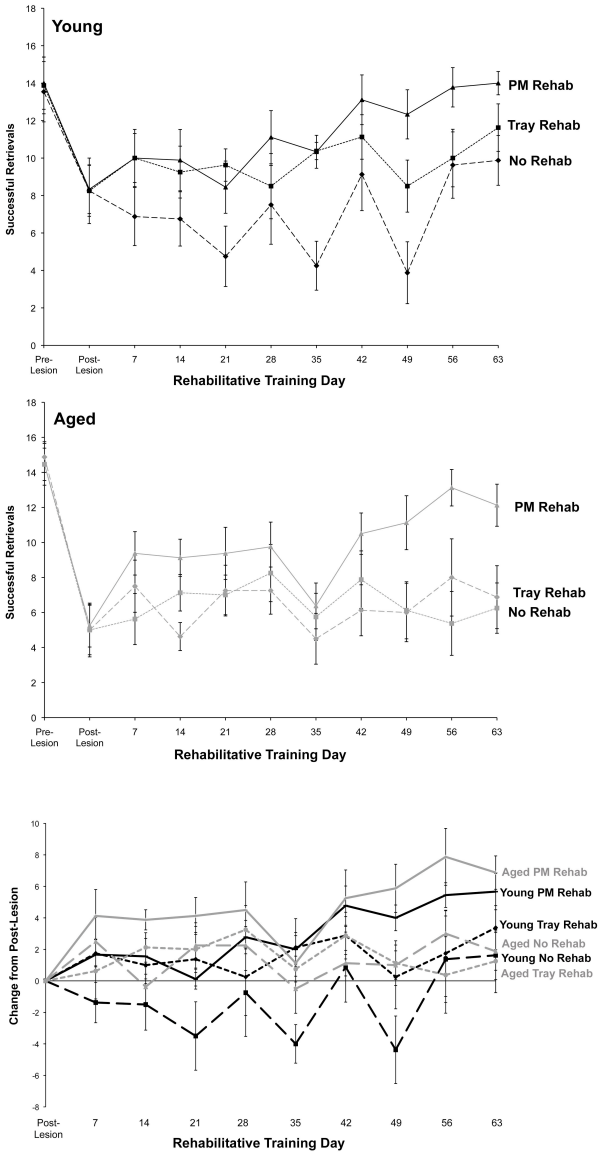


Figure 5.3 Both young and aged mice improved post-operative reaching success with rehabilitative training on the Pasta Matrix Reaching Task. (A) Young mice improved function with post-operative rehabilitative training on both the Tray and Pasta Matrix Reaching Tasks. (B) Aged mice improved function only after rehabilitative training on the Pasta Matrix Reaching Task. Performance of the Tray Rehab and No Rehab groups was similar. (C) Change from post-operative performance (calculated as the initial post-lesion success subtracted from each following probe trial) was used as a measure of post-operative deficits.

5.4.2.2 Aged mice

Pre-operative performance on the Pasta Matrix Reaching Task was similar in young and aged mice ($t(47)=-0.78$, $p=0.44$). Like young mice, aged mice that received Pasta Matrix Reaching as rehabilitative training improved reaching success compared to aged mice that received no rehabilitative training. When the results of weekly probe trials were compared across training conditions, there was no effect of rehabilitative training in general ($F(1,22)=1.26$, $p=0.27$) nor a significant day x group interaction ($F(10,220)=1.02$, $p=0.43$, PM Rehab and Tray Rehab vs. No Rehab). Pasta Matrix training resulted in a significant group effect ($F(1,14)=4.63$, $p=0.049$) but no day x group interaction ($F(10,140)=1.56$, $p=0.13$, PM Rehab vs. No Rehab). Tray Reaching training did not result in an effect of group ($F(1,14)<0.001$, $p=0.99$) or day x group interaction ($F(10,140)=1.03$, $p=0.42$, Tray Rehab vs. No Rehab). These results indicate that only Pasta Matrix Reaching was effective in improving function in aged mice compared to mice that did not receive rehabilitation.

Unlike young mice, movement analysis indicated that aged mice showed far less improvement in the manner in which they reached following rehabilitative training. In comparison to control animals, Tray Reaching training did not results in any significant group effects in the frequency of atypical movements (Fs=0.01-0.87, ps=0.39-0.92) or day x group interactions (Fs=0.49-0.69, ps=0.73-0.89). However, Pasta Matrix training resulted in significant group effects for advancing ($F(1,5)=9.56$, $p=0.03$) and opening the digits ($F(1,5)=6.71$, $p=0.049$), but no significant day x group interactions (Fs=0.53-1.17,

*p*s=0.34-0.86) when compared to untrained controls. Additionally, there was a significant group effect in advancing when the two rehabilitative training groups were compared ($F(1,6)=8.04$, $p=0.03$, Tray Rehab vs. PM Rehab), but no day x group interactions (*Fs*=0.96-1.46, *ps*=0.18-0.49). Similar to overall success rates, Pasta Matrix training, but not Tray Reaching training, was effective in improving qualitative reaching performance in aged mice. However, these improvements were not as great as those seen following rehabilitative training in young mice.

5.4.2.3 Comparison of behavioral improvements in young and aged mice

Aged mice, which had larger lesions than young mice, were more impaired in the first post-operative probe session ($t(47)=2.85$, $p=0.007$) and at the end of the 9 weeks of rehabilitative training compared to young mice ($t(47)=2.91$, $p=0.005$). In addition to impairments in overall success rate, the quality of reaching was more abnormal in aged mice compared to young mice. Specifically, aged mice were more impaired (measured by the frequency of atypical movements) in opening the digits ($t(22)=-2.31$, $p=0.03$), breaking the pasta ($t(22)=-3.52$, $p=0.002$), and withdrawing the pasta ($t(22)=-2.32$, $p=0.03$). Even after the rehabilitative training period, aged mice continued to differ from young mice in opening the digits ($t(22)=-2.56$, $p=0.02$), despite not differing from young animals prior to lesion induction in any movement component (*t*s=-1.09-1.81, *p*s=0.09-0.91).

To assess the effect of age on behavioral improvement in each rehabilitative training group, we directly compared the change from post-operative performance of young and aged mice (Fig 5.3C). There were no effects of age in the No Rehab ($F(1,14)=2.35$, $p=0.15$), Tray Rehab ($F(1,14)=0.25$, $p=0.63$), or PM Rehab conditions ($F(1,16)=0.30$, $p=0.59$). There were also no day x age interactions for No Rehab, Tray Rehab or PM Rehab conditions ($F(9,126)=1.48$, $p=0.16$, $F(9,126)=0.77$, $p=0.64$, $F(9,144)=0.97$, $p=0.47$, respectively). The results indicate that rehabilitative training on the Pasta Matrix Task had a similar effect on behavioral improvement in young and aged mice. Even though aged mice were initially more impaired than aged mice, they were able to improve to a similar magnitude as young mice following task-specific rehabilitative training.

5.4.3 Other behavioral measures

The performance of young and aged mice on other tasks of sensorimotor behavior was compared. Both young and aged animals showed no behavioral differences due to rehabilitative training condition, and thus all conditions were combined for analysis to focus on the age differences both prior to lesion and post-operatively.

5.4.3.1 Capellini Handling Test

The number of adjustments with each paw, the number of atypical eating behaviors, and the total time to eat were averaged over three pasta pieces eaten by each animal. The number of adjustments made with either paw did not change significantly

following lesion induction or post-operative training for young or aged animals. Compared to young mice, aged mice ate pasta in a more atypical way, both before and after injury ($F(1,42)=13.35$, $p=0.001$; Fig. 5.4A), but there was no significant post-lesion

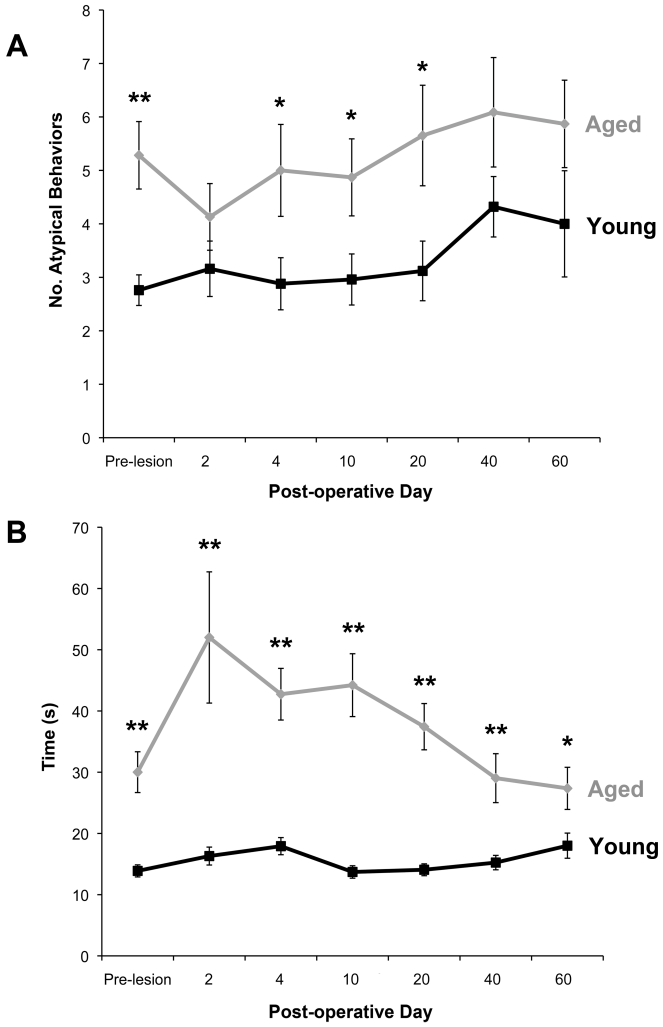


Figure 5.4 Behavioral performance on the Capellini Handling Test. (A) Aged mice used more atypical handling behaviors during pasta eating compared to young mice. *p<0.05, Young vs. Aged; **p<0.01, Young vs. Aged. (B) Aged mice took longer to eat than young mice, both before and after injury. Additionally, eating time was significantly slower for aged mice on post-operative days 2, 4 and 10, compared to pre-lesion performance. *p<0.05, Young vs. Aged; **p<0.01, Young vs. Aged.

increase in the number of atypical behaviors in either age group. Aged mice took significantly more time to eat than young mice both before and after injury ($F(1,42)=42.16$, $p<0.001$, Fig. 5.4B). While the eating time of young mice did not change following lesion induction, post-lesion eating time of aged mice was significantly slower ($F(6,252)=4.06$, $p=0.001$). By post-operative day 20, aged animals' eating time returned to pre-operative levels.

5.4.3.2 Ladder Rung Walking Test

Videotapes were scored using slow motion analysis for the total number of footfaults with the contralesional paw and the total time to cross the ladder. All animals made more errors on the ladder with the irregularly patterned rungs that with the evenly spaced rungs. On the even ladder, there was a significant effect of age ($F(1,43)=11.35$, $p=0.002$) and a significant day x age interaction ($F(6,258)=7.93$, $p<0.001$). On the irregular ladder, there was a significant day x age interaction ($F(6,258)=4.03$, $p=0.001$) but no effect of age ($F(1,43)=0.37$, $p=0.55$). Aged mice made significantly more post-operative errors on both the ladders compared to pre-operative performance and performance of young animals (Fig. 5.5A,B). These errors were resolved by post-operative day 10 on the irregular ladder and day 20 on the even ladder. Additionally, young mice were significantly slower in crossing the ladder with evenly spaced rungs ($F(1,43)=5.30$, $p=0.03$). There was no difference between the time taken by young and

aged mice to cross the irregularly patterned ladder, although all mice crossed this ladder slower than the evenly patterned (Fig. 5.5C).

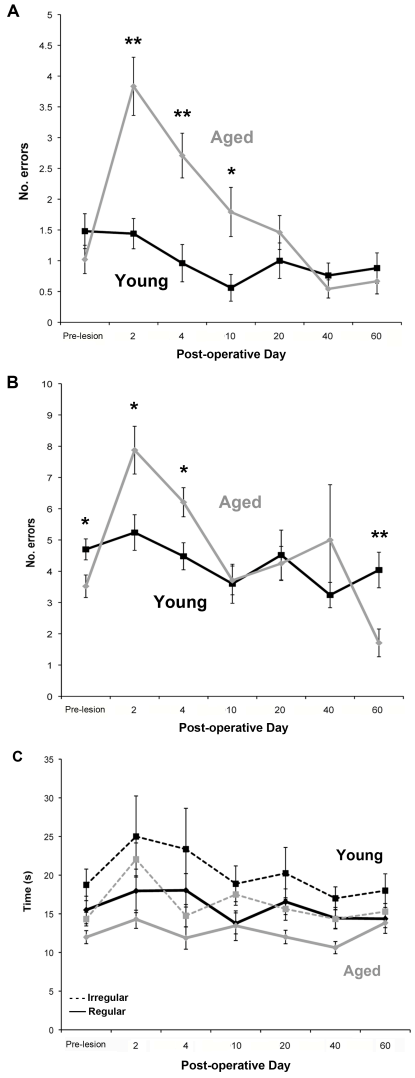


Figure 5.5 Behavioral performance on the Ladder Rung Walking Test. (A) On the regularly patterned ladder, aged mice made significantly more post-operative stepping errors with the contralesional forepaw compared to pre-operative and young animals' performance. This deficit was resolved by post-operative day 20. *p,0.05. Young vs. Aged. **p<0.01, Young vs. Aged. (B) Aged mice also made more errors on the irregularly patterned ladder, but this deficit was resolved by day 10. *p,0.05. Young vs. Aged. **p<0.01, Young vs. Aged. (C) All mice took longer to cross the irregularly patterned ladder compared to the regularly patterned ladder.

5.4.3.3 Bilateral Tactile Stimulation Test

The time to contact and remove each stimulus was averaged across 3 trials per testing day for each animal. There was a significant day x age interaction ($F(6,258)=2.44$, $p=0.03$) and a nearly significant effect of age ($F(1,43)=2.95$, $p=0.09$) in the time to contact the contralesional stimulus. There was a significant effect of age ($F(1,43)=13.51$, $p=0.001$) and a significant day x age interaction ($F(6,258)=5.61$, $p<0.001$) in the time taken to remove the contralesional stimulus. Aged mice show a transient impairment in sensory function and a relatively long-lasting impairment in motor function of the contralesional paw. Aged mice take significantly longer to contact the contralesional stimulus at 2 days post-lesion (Fig. 5.6A) and are impaired in stimulus removal until post-operative day 20 (Fig. 5.6B). Rehabilitative training had no beneficial effect on improving contact or removal times in young or aged animals.

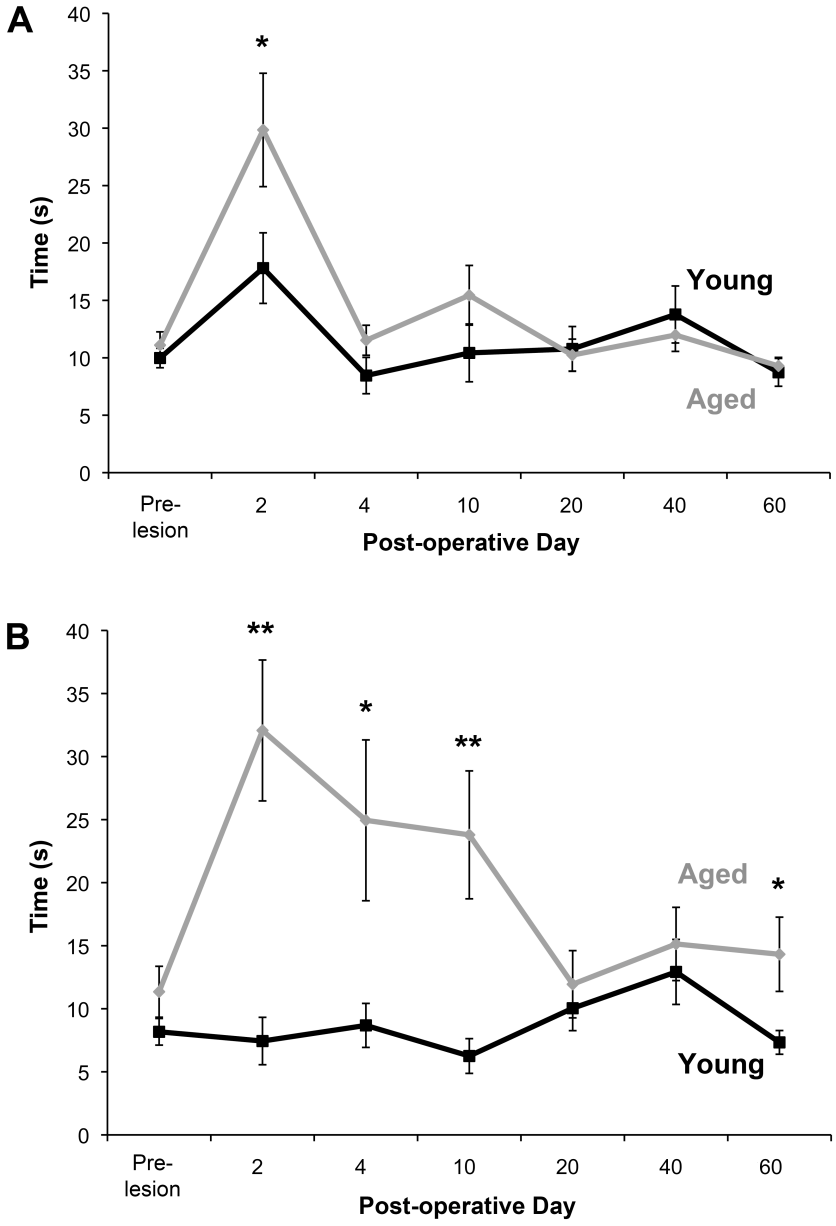


Figure 5.6 Behavioral performance on the Bilateral Tactile Stimulation Test. (A) Aged mice had a transient increase in the time taken to contact the stimulus on the contralesional paw. *p<0.05, Young vs. Aged. (B) Aged mice had a deficit in removing the stimulus from the contralesional paw that persisted until post-operative day 20. *p<0.05, Young vs. Aged; **p<0.01, Young vs. Aged.

5.4.6 Intracortical microstimulation mapping

There were no significant group differences found in the areas of individual or combined forelimb representations within the CFA or RFA of young and aged mice (Fs=0.13-2.72, ps=0.12-0.88). However, young mice that were post-operatively trained on the Pasta Matrix Reaching Task had a trend towards an enlargement of the RFA compared to untrained controls ($F(1,8)=4.16$, $p=0.08$; Fig. 5.7A). The slight enlargement of the RFA was due to combined increases in proximal and distal movement representations. Mice that received Tray Reaching training were not significantly different from controls ($F(1,5)=2.00$, $p=0.23$) or Pasta Matrix trained mice ($F(1,8)=1.64$, $p=0.24$). There were no significant differences in the ratio of proximal to distal representations within the CFA ($F(1,15)=0.95$, $p=0.35$; Fig. 5.7B) or RFA ($F(1,8)=1.67$, $p=0.24$) of Pasta Matrix trained mice compared to untrained controls, nor between Tray Reaching trained and control mice (CFA: $F(1,14)=1.33$, $p=0.27$; RFA: $F(1,5)=0.10$, $p=0.77$). The ratios within the CFA ($F(1,16)=0.01$, $p=0.91$) and RFA ($F(1,8)=1.55$, $p=0.25$) were also not significantly different between young Pasta Matrix and Tray Reaching trained mice.

There were no significant group differences in the areas of individual or combined forelimb representations in the aged CFA or RFA (Fs=0.008-2.24, ps=0.13-0.99). There were also no differences in the ratio of proximal (elbow and shoulder) to distal (wrist and digit) movement representations within the CFA or RFA of untrained control mice compared to Tray Reaching (CFA: $F(1,14)=0.61$, $p=0.45$; RFA: $F(1,6)=3.57$, $p=0.12$) and Pasta Matrix Reach trained (CFA: $F(1,14)=0.12$, $p=0.74$; RFA: $F(1,4)=0.56$, $p=0.51$)

young mice. There were also no significant differences in ratios between mice that received Pasta Matrix or Tray Reaching rehabilitative training (CFA: $F(1,13)=1.59$, $p=0.23$; RFA: $F(1,7)=0.00$, $p=1.00$).

Although aged mice have larger lesions than young mice, they retain a remarkable amount of cortical map area (Table 5.2). In comparison to the mean forelimb representation areas collected from intact animals in Chapter 3, young and aged mice both lose approximately half of the total CFA area following ischemic lesions (1.68 ± 0.08 vs. 0.87 ± 0.09, young intact vs. young ischemic mice; 2.01 ± 0.14 vs. 0.88 ± 0.10, aged intact vs. aged ischemic mice). However, aged mice may be losing more RFA area due to ischemia than young mice. While young mice likely do not lose significant RFA area (0.18 ± 0.01 vs. 0.22 ± 0.05, young intact vs. young ischemic mice), aged mice, which have a larger RFA to begin with (Chapter 3), may be losing some RFA area due to the lesion (0.24 ± 0.02 vs. 0.17 ± 0.04, aged intact vs. aged ischemic mice). However, the number of aged animals with a discernable RFA in this study (n=10 out of 24 total) was not different from young animals (n=12 out of 25 total; $\chi^2(49)=0.20$, $p=0.90$). It should be noted that these results are from different studies in different populations of age-matched animals, and only a within-animal study would provide the proper support for any significant changes in loss of map area following ischemia.

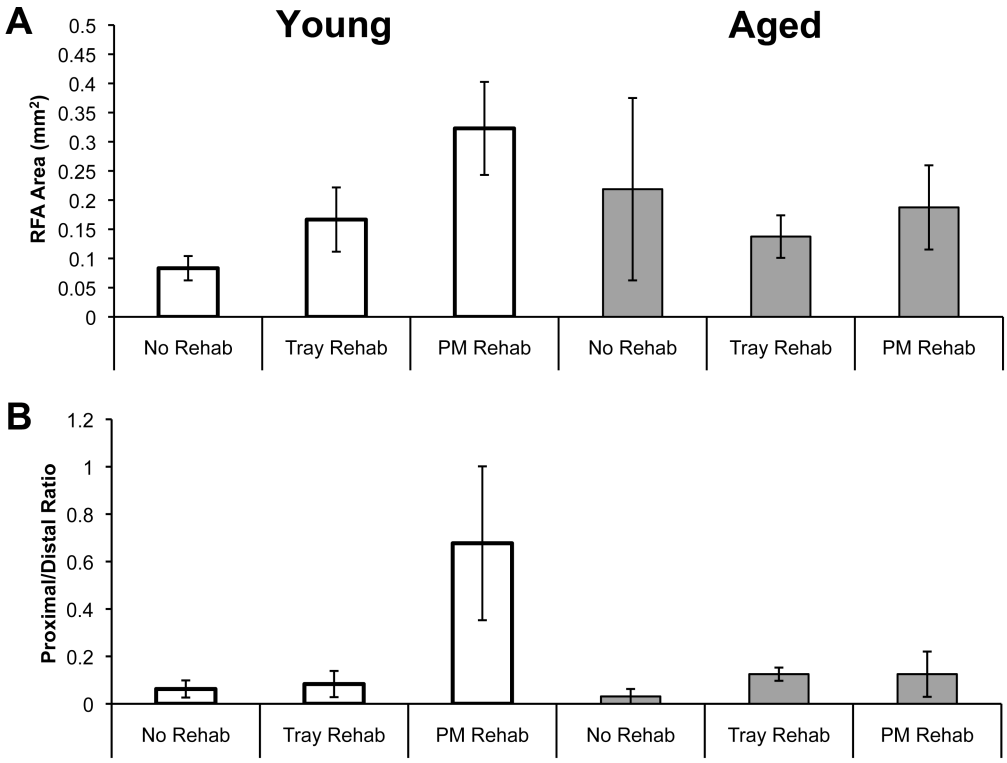


Figure 5.7 ICMS-evoked maps of the RFA. (A) Areal extents of the RFA across ages and training conditions. (B) The ratio of proximal to distal forelimb representations within the RFA across ages and training conditions.

Table 5.2 Proportions of remaining cortical forelimb representations within CFA

		% Shoulder	% Elbow	% Wrist	% Digit	Summed Area (mm^2)
Young	No Rehab	15.27 (6.88)	17.79 (9.33)	12.92 (9.26)	54.01 (11.95)	0.75 (0.17)
	Tray Rehab	21.09 (11.69)	23.84 (10.02)	21.71 (5.79)	33.37 (11.61)	0.73 (0.08)
	PM Rehab	25.31 (8.85)	19.25 (5.01)	18.66 (4.84)	36.77 (9.26)	1.08 (0.17)
Aged	No Rehab	53.87 (12.58)	8.40 (3.92)32.14	12.55 (4.55)	25.18 (9.02)	0.96 (0.22)
	Tray Rehab	20.00 (7.35)	21.99 (13.14)	32.14 (10.19)	25.87 (11.16)	0.83 (0.21)
	PM Rehab	53.09 (14.75)	6.20 (3.34)	10.71 (8.81)	30.00 (9.79)	0.85 (0.08)

All data are means ± SEM.

5.5 Discussion

One of the most common and unavoidable risk factors for stroke is age (Roger et al., 2011). Following focal ischemic lesions, aged mice exhibit deficits similar to those seen in human stroke survivors, including loss of dexterous use of the forelimb for reach-to-grasp or food handling tasks, impairments in coordinated forelimb use and hyperreliance on the ipsilesional or "good" limb. Structured, task-specific rehabilitative training seems to be a key factor in regaining function of the contralesional limb after stroke in both young and aged animals. In human stroke survivors, task-specific

rehabilitation has been shown to improve motor function (i.e. dexterity, range of motion) of the upper extremities (Smith et al., 1999; Whitall et al., 2000; Galea et al., 2001; Winstein et al., 2001) and prevent compensation with the less-impaired side of the body (Michaelson et al., 2006; Lurn et al., 2009). Even when rehabilitation was initiated over 1 year post-stroke, task-specific training resulted in behavioral improvements (Dean and Shepherd, 1997). Our results suggest that, especially in old age, task-specific rehabilitative training is one of the best ways to induce behavioral improvement of the upper extremities.

In both humans and rodents, the effects of ischemic lesions become more devastating in old age, resulting in greater impairments, more difficulty regaining function with rehabilitative training, and less spontaneous recovery in the absence of training. Larger ischemic lesions have previously been shown in many rodent studies (for examples, see Davis et al., 1995, Kharlamov et al., 2000, and Merritt et al., 2009). Based on the results of the current study, the larger lesions of aged mice may be a factor in the poor post-operative motor performance compared to young mice. During ET-1 infusion surgeries, we attempted to equalize the final volume of lesions between young and aged animals by infusing a smaller volume of ET-1 into the SMC of aged mice during lesion induction. Even with the decreased dose of vasoconstricting peptide, lesions were significantly larger in the aged mouse brain compared to the young brain. These results suggest that the aged brain is more sensitive to ischemia, and it's the increased damage caused by this sensitivity that is creating greater behavioral deficits and the absence of

rehabilitative training-induced reorganization within the motor cortical representation rather than a lack of plasticity of the aged motor cortex.

Prior studies have shown that rats with large ET-1 induced lesions of the SMC show greater deficits in sensorimotor function of the contralesional limb, and show less dendritic plasticity in the contralateral homotopic cortex following reach training with the ipsilesional, "less affected" limb (Hsu and Jones, 2006). Gharbawie et al. (2007) showed that lesions of the entire motor cortex resulted in greater reaching deficits compared to lesions of the caudal or rostral extents of the motor cortex. Additional lesions of contralateral homotopic or perilesion cortex worsen deficits. It is thought that these areas reorganize following MI damage to support motor performance. It would make sense that the more area damaged by an ischemic lesion, the less intact area there is to take over the lost functions.

In young rats and nonhuman primates, relearning of skilled hand movements through rehabilitative training results in the maintenance of surviving motor cortical representations and remapping of lost representations onto perilesion cortex (Nudo et al., 1996b, Kleim et al., 2003b). In young mice, somatosensory maps have been shown to reorganize into surrounding intact motor cortical territory following lesions of the forelimb sensory representation (Brown et al., 2009; Sigler et al., 2009). The results of the current study suggest that rehabilitation-induced plasticity in the motor cortex of the young mouse may function in a similar way. Young mice that are post-operatively trained on the Pasta Matrix Reaching Task regain reaching function to pre-operative success levels after 6 weeks of rehabilitation. ICMS-evoked maps in young Pasta Matrix trained

mice show a slight expansion in the size of the RFA. It has been proposed that the RFA of the rat brain is analogous to the premotor or supplementary motor areas of the monkey brain (Neafsey and Sievert 1982; Barth et al. 1990; Rouiller et al. 1993; Dancause et al. 2006; Eisner-Janowicz et al. 2008). In squirrel monkeys, lesions of the MI hand area result in enlargement of the hand representation within the ventral premotor cortex (PMv; Frost et al., 2003). Following lesions of both the MI and PMv, there is an enlargement of the hand representation in the supplementary area (SMA; Eisner-Janowicz et al., 2008). In both areas, the enlargement is proportional to the amount of hand representation damaged by the lesion. Evidence from Gharbawie et al. (2007) implicates the RFA as a potential site for plasticity in the rodent brain following CFA lesions. Rats with complete lesions of the motor area, inclusive of CFA and RFA, are more impaired than animals that only have damage to the caudal extent of the motor cortex, inclusive of CFA. The current study extends this analogy to the mouse brain and further supports the role of the RFA as a non-primary motor region.

Although aged animals regained reaching ability following rehabilitative training on the Pasta Matrix Reaching Task, we failed to find any changes in ICMS-evoked motor maps. This could possibly be explained by the larger lesions seen in aged brains resulting in greater damage to the areas that would normally compensate for lost functions. Alternatively, it is feasible that plasticity in the aged brain could occur at a later timepoint, following a longer duration of rehabilitative training. Cotman and Scheff (1979) showed that it takes nearly 4 times the duration of time for synaptogenesis to occur in the aged dentate gyrus following fibrial transection compared to the rate of

reinnervation in the young hippocampus. Future studies can take advantage of transgenic mouse strains to determine the potential for rehabilitative training to induce plasticity in the motor cortex using two-photon imaging and light-based motor mapping in mice expressing fluorescent proteins and/or channelrhodopin in layer V neurons of motor cortex (Ayling et al., 2009). Overall, the results of the current study show that the aged brain undergoes more damage following ischemia, but aged animals are able to recover function to a similar extent as young animals if provided with task-specific rehabilitative training.

Chapter 6: Discussion

6.1 Summary

These dissertation studies provide support for healthy older adults' abilities to acquire new motor skills and reacquire motor skills following focal ischemic lesions. However, the aged brain responds to motor skill learning with altered motor cortical map plasticity and is more sensitive to the damaging effects of cerebral ischemia. These studies suggest that despite age-related changes in both motor behaviors and the function of the motor cortex, healthy older adults maintain a strong capacity for learning new motor tasks, both before and after brain injury.

The forelimb motor representation of mice resembled that of other species commonly used for studies of motor skill learning, such as rats and monkeys (Chapter 2). Mice were similar to rats in their ability to learn new motor skills (Chapter 3), but the motor representation of the mouse differed from that of rats in the relative size of the digit representation. While digit is not typically found in rat motor maps, and when it is, it is restricted to the rostral motor cortex (RFA), nearly half of the forelimb motor cortical representation was composed of digit representation in the mouse (Chapter 2). When young mice were trained on a skilled reaching task, there was an increase in the ratio of proximal (elbow) to distal (wrist and digit) forelimb representations. Mice that were trained for a longer duration on the same task did not show an increase in the proximal to distal forelimb ratio (Chapter 3). These findings agree with those of Molina-Luna et al. (2008) who showed that reorganized motor areas return to baseline organization

following a period without practice on the task. Our results support the idea of motor map plasticity as a transient phenomenon and further show that ongoing practice on a task does not result in maintenance of map reorganization.

Following endothelin-1 (ET-1) lesions of the sensorimotor cortex, young mice exhibited deficits in skilled reaching behavior and other sensorimotor behaviors, such as coordinated forepaw use during walking (Chapter 4). Compared to young mice, aged mice were found to be more impaired on motor tasks following sensorimotor cortical stroke and remain impaired for a longer duration (Chapter 5). Even though aged mice received a smaller dose of vasoconstricting peptide, lesions were significantly larger in the aged mouse brain compared to the young brain. This adds further support to findings that the aged brain is more sensitive to the damaging effects of ischemia, and this sensitivity results in greater tissue loss and more severe behavioral deficits. At least as measured by these studies, it seems that differences in lesion size may contribute to the poorer prognosis of older stroke survivors.

Despite larger lesions in the aged brain, structured, task-specific rehabilitative training seems to be particularly effective in improving function of the contralesional limb after stroke in both young and aged animals. Young mice that were post-operatively trained on the Pasta Matrix Reaching Task regained reaching function to pre-operative success levels after 6 weeks of training and ICMS-evoked maps in young Pasta Matrix trained mice showed an expansion in the size of the RFA. In aged mice, although rehabilitative training on the Pasta Matrix Reaching Task resulted in improvements in reaching behavior, no map changes were detectable by ICMS after 9 weeks of training.

These results suggest that despite altered motor cortical map plasticity, older adults are able to reacquire previously learned motor skills if given appropriate rehabilitative training.

6.2 The organization of the mouse motor cortex parallels that of other species commonly used to study motor cortical plasticity

Like humans, monkeys and rats, the results of Chapters 3 and 4 revealed that mice are extremely dexterous with their forepaws and digits. This dexterity was evidenced by their ability to learn and perform motor tasks and reflected in the topographical organization of their motor cortex (Chapter 2). The C57BL/6 mouse motor map resembles, in its general organization, those found in other species, such as rats (Donoghue and Wise 1982), cats (Asanuma and Sakata 1967), and monkeys (Nudo et al. 1996), as well as those described in other strains of mice (Li and Waters 1991; Pronichev and Lenkov 1998). Like rats, we found that a majority of mice have two distinct forelimb representations: a large caudal map (CFA) and a smaller rostral map (RFA). The most striking difference in the forelimb motor map of mice compared with rats is the prominence and location of the digit representation. The digit representation in the mouse motor cortex is large, whereas digit movements are rarely evoked with ICMS in the rat motor cortex (Hall and Lindholm 1974; Donoghue and Wise 1982; Kleim et al. 1998) and when they are, they are usually found in the RFA, and rarely in the CFA (Kleim et al. 1998). The area of the map devoted to a movement is thought to correspond with the dexterity of that movement (Monfils et al. 2005) and although it is tempting to speculate

that the difference in digit representations corresponds to species-related differences in manual dexterity, it cannot be ruled out that the same ICMS parameters and anesthetics result in different neural activating effects across these species and that this contributes to a more sensitive detection of digit representations in the mouse compared with the rat motor cortex. Futhermore, the ratio of proximal to distal representations is highly similar between rats and mice. However, in mice, the elbow representation appeared to make up a smaller portion of the total forelimb representations than it does in rats (Kleim et al., 1998).

6.3 The role of the motor cortex in long-duration motor skill practice

Skilled learning in rats has been related to increases in the area of distal forelimb movement representations, primarily wrist representations, at the expense of proximal movement representations, primarily elbow representations. In untrained rats, approximately 40% of the forelimb map is comprised of distal forelimb representation whereas after substantial training in a skilled reaching task, the distal representation comprises approximately 75% of the rat forelimb map (Kleim et al. 1998). In the second experiment (Chapter 3), we found an increase in the ratio of proximal (elbow) to distal (wrist and digit) forelimb representations in the CFA when mice were trained for 1 month. After a longer duration of training, the ratio of elbow to distal representations had returned to baseline. There are at least two possibilities for why the increase in the proximal to distal ratio is transient. The first is that after a longer duration of training, the reorganized region of motor cortex is no longer required for performance of the task and

the map returns to baseline (as found by Molina-Luna et al., 2008). However, this possibility conflicts with studies that have shown loss of motor skill following lesions of the sensorimotor cortex (Kleim et al., 2003; Adkins et al., 2004; Gharbawie et al., 2007) as well as findings of the long-term maintenance of new spines generated early in learning (Xu et al., 2009). A more likely possibility is that ICMS is detecting a transient stage in the long-term reorganization of the motor cortex that subserves the maintenance of the skill. Skilled learning-induced plasticity in the forelimb movement representation is thought to reflect the recruitment of neurons in the cortical territory devoted to producing novel movement sequences necessary for performing a newly learned motor skill (Keller, 1993). The interconnected nature of the motor representation is thought to provide the flexibility necessary to modify the existing network to accommodate this behavioral change (Sanes and Donoghue, 2000). In support of this, the representations of the digits, wrist, elbow and shoulder overlap within the forelimb representation area (Chapter 2; Tennant et al., 2011) as well as overlapping with the body representations that border the forelimb representations (Chapter 2; Tennant et al., 2011). The representation areas within the motor cortex are interconnected via layer II cortico-cortico connections, and plasticity of these connections is a proposed mechanism of motor map reorganization (Rioult-Pedotti et al., 2007).

It has been proposed that the process underlying reorganization of the motor map may be partially mediated by an LTP-like mechanism (Monfils and Teskey, 2004) and that motor skill training results in a long-term increase in the synaptic modification range that allows for additional LTP in the motor cortex (Rioult-Pedotti et al., 2007). This

potentiation, along with a transient net increase in spines seen during the early stages of motor skill learning (Xu et al., 2009), may result in the transient shift of movements most easily elicited by ICMS stimulation. There may be a prolonged time period in which the processes of synaptic pruning and potentiation are still ongoing and the resetting of the motor map may coincide with the full establishment of the circuitry underlying the ability to continue to perform the skill proficiently. In other words, the resetting of the forelimb motor map organization to baseline is unlikely to indicate a reversal of the plasticity, and it might indicate that the plastic changes are being more permanently incorporated into the motor cortical network, possibly through the addition of spines and/or synapses. Kleim et al., (2002) showed that the number of synapses per neuron increased in the CFA, but not the RFA or hindlimb area, of rats that were trained on a skilled reaching task. Thus, synaptogenesis was restricted to cortical motor representations that underwent reorganization. Additionally, although we found that the forelimb cortical motor map of mice returned to control levels after a longer duration of practice on the task, stimulation thresholds decreased for wrist and digit movements in this late time period. It is possible that lower movement thresholds are associated with increased synaptic efficacy (Monfils et al., 2005), and that the lower thresholds seen after extended training are due to an area-specific lasting synaptic potentiation that is maintained after the map has reset to baseline.

In support of motor map organization resetting following long-term motor skill practice in humans, fMRI studies show significantly less activation of the primary and secondary motor areas of professional pianists in bimanual and unimanual performance

tasks compared to non-musician controls (Jancke et al., 2000; Krings et al., 2000). These findings have been interpreted as suggesting that fewer neurons need to be recruited to perform the same skilled movements in long-term pianists (Krings et al., 2000). Additionally, when pianists are learning a novel finger-tapping task, fMRI-measured activation of primary and secondary motor areas is rapidly attenuated compared to non-musicians (Hund-Georgiadis and von Cramon, 1999). Pearce et al., (2000) found that professional badminton players show decreases in the amount of TMS current required to evoke a movement compared to social players or non-players. This finding parallels our results that ICMS evoked movements at lower stimulating currents in long-duration reach trained mice. Human studies also support that the motor cortex mediates the complex movement sequences used in manual skills and the learning of these skills. For example, Gentner et al. (2010) showed that movement sequences that approximate instrument playing in professional violinists could be induced with TMS. In rats, a different from of ICMS from the one used in these dissertation studies, long-duration ICMS, produces complex, multi-joint movements which approximate reaching and grasping movements (Ramanathan et al., 2006). Reorganization of complex movement representations was seen following focal electrolytic lesions of the CFA and rehabilitative training, but not following short duration motor skill training in intact animals. It is possible that following long-term practice on a skilled motor task, the organization of complex movement representations may differ between animals trained for long and short durations.

6.4 Learning new skills late in life

Evidence from studies of the somatosensory cortex in aged rats show that the forelimb somatosensory map goes through a gradual decrease in the size of glabrous receptive fields and subsequent increase in the size of non-glabrous receptive fields (Coq and Xerri, 2000). This change coincides with impairments in walking behavior (David-Jürgens et al., 2008) and is somewhat attenuated when aged animals are housed in enriched environments from weaning. The motor cortex of aged mice seems to go through a somewhat similar process (Chapter 3). There was a decrease in the area of digit representations and an increase in wrist representations in the aged CFA, and the aged RFA was larger than the young RFA in total area.

It has been proposed that the RFA of the rodent brain may be homologous to the premotor or supplementary motor areas of the primate brain (Neafsey and Sievert 1982; Barth et al. 1990; Rouiller et al. 1993; Dancause et al. 2006; Eisner-Janowicz et al. 2008). Post-operative motor mapping studies in rats (Conner et al., 2005) and monkeys (Liu and Rouiller, 1999) have shown evidence that the RFA of rats and premotor cortex of monkeys undergo plastic changes following damage to primary motor areas, but not during motor skill learning in the intact brain (Kleim et al., 1998). Additionally, human fMRI studies have shown evidence of increased activation (measured as increased hemodynamic response) in premotor, supplementary motor, sensory, and cognitive areas during motor performance in elderly people (Heuninckx et al., 2005; 2008).

Although these dissertation studies failed to find plasticity in motor cortical representation areas in aged mice, there may be additional mechanisms, such as increases

in dendritic spines, synapses, astrocytes, or vasculature, supporting skill learning within this area (Kleim et al., 2002a,b; Xu et al., 2009; Kim and Jones, 2010). *In-vivo* studies using young fluorescent protein-expressing mice have shown that motor learning results in nearly immediate increases in spine formation and a more protracted period of selective spine elimination within the motor cortex (Xu et al., 2009). The similarity in the behavioral changes following motor skill learning and rehabilitative training in aged vs. young mice alludes to some correspondence of changes in motor cortex plasticity. As of yet, there is no research on the rate of spine formation and elimination concurrent with motor skill learning in aged mice and it remains unknown if the additional activation of motor and sensory areas in the aged human brain are supported by structural plasticity. It is feasible that the time course measured in this study missed the transient motor map reorganization stage of motor cortical plasticity in the aged brain or that further reorganization of the aged motor map could occur following an even longer duration of reach training. Cotman and Scheff (1979) showed that it takes nearly 4 times as long for synaptogenesis to occur in the aged dentate gyrus following fibrial transection compared to the rate of reinnervation in the young hippocampus. However, given that motor learning in older humans involves more non-primary motor and non-motor areas compared to young adults, it could also be that the motor cortex does not play as large a role in motor learning in aged animals as it does it young animals. The loss of skill following sensorimotor cortical lesions in aged animals could be due not only to damage of primary motor areas but also disconnection from areas that play a larger role in motor skill learning and production in aged animals. The ability of aged mice to learn a motor

skill despite having deficits on other sensorimotor tasks, as shown in Chapter 3, suggests that there may be a dissociation between skill learning and motor performance in the intact aged brain as well.

6.5 Implications for rehabilitation of elderly stroke survivors

One of the most common and unavoidable risk factors for stroke is age (Roger et al., 2011). Although evidence points to a link between age and decreased prognosis for recovery following stroke (Pohjasvaara et al., 1997; Patrick et al., 2001; Kammersgaard et al., 2004; Rojas et al., 2007; Divani et al., 2011), the majority of preclinical stroke studies are conducted in young animals. This choice may be due in part to time- and cost-saving measures. However, when mice are utilized as a model of aged stroke, the requisite time involved in aging the animals to a clinically relevant age is only 18 months (Barreto et al., 2010), as opposed to 24 months in rats (Markus et al., 2005; Alaverdashvili and Whishaw, 2010) and 15 years in primates (Moore et al., 2010).

Like aged humans, aged mice were found to be more impaired on motor tasks following sensorimotor cortical lesions and remained impaired for a longer duration than young mice (Chapter 5). Volume measurements of remaining cortical area within the SMC of young and aged mice showed that lesion size was significantly larger in aged mice. Prior studies have shown that rats with large lesions of the forelimb area of the SMC show greater deficits in sensorimotor function of the contralesional limb (Hsu and Jones, 2006) and lesions of the entire motor cortex result in greater reaching deficits compared to lesions of the caudal or rostral extents of the motor cortex (Gharbawie et al.,

2007). Based on the results of these dissertation studies, it seems that the greater behavioral deficits seen in older animals following focal ischemic lesions are due primarily to larger lesion sizes. Previous studies have also found an age-related increase in infarct size (e.g. Davis et al., 1995; Merritt et al., 2009), but there is evidence that aged animals do not always have larger lesions (e.g. Popa-Wagner et al., 1998; Badan et al., 2003). Some of the mechanisms that have been found to contribute to larger infarcts and/or greater behavioral impairments following ischemic lesions in aged animals include accelerated formation of the glial scar (Badan et al., 2003; Popa-Wagner et al., 2006), increased apoptosis (Gozal et al., 2003), greater oxidative damage (Charmichael, 2006), and increased fragility of aged blood vessels (Hajdu et al., 1990). In our study, even though aged mice received a smaller dose of vasoconstricting peptide, lesions were significantly larger in the aged mouse brain compared to the young brain. This suggests that the aged brain is more sensitive to the damaging effects of ischemia and or to the vasoconstricting effects of ET-1, and this sensitivity results in greater tissue loss and more severe behavioral deficits. Studies of human vasculature show that endogenous ET-1 increases in aged vasculature (Goel et al., 2010; Seals et al., 2011), which may contribute to increased vasoconstriction and an inability to reperfuse brain tissues following ischemia (Donato et al., 2009). Even despite larger lesions in the aged brain, structured, task-specific rehabilitative training seems to be particularly effective in regaining function of the contralesional limb after stroke in both young and aged animals. These results suggest that differences in lesion size, rather than a lack of ability to profit

by rehabilitative training, may contribute to the poorer prognosis of older stroke survivors.

In young rats and nonhuman primates, relearning of skilled hand movements through rehabilitative training results in the maintenance of surviving motor cortical representations and reorganization of damaged motor areas onto perilesion cortex (Nudo et al., 1996b; Kleim et al., 2003b). In young mice, somatosensory maps reorganize into surrounding intact motor cortical territory following lesions of the forelimb sensory representation (Brown et al., 2009; Sigler et al., 2009). This reorganization is supported by expansive increases in dendritic spine turnover and vascular remodeling in perilesion cortex (Brown et al., 2007). However, these dissertation studies have failed to find evidence of reorganization of motor cortical representations in aged animals. It is not clear from our results whether the lack of motor map plasticity in this study was due to lesion size or age-related differences in the way the aged motor cortex responds to skill learning.

6.6 The RFA as a potential substrate for rehabilitation-induced reorganization in the mouse brain

The results of these dissertation studies indicate that the RFA in the young mouse brain undergoes reorganization and expansion following focal ischemic lesions of the CFA. Rehabilitative training results in behavioral improvement to pre-operative levels in young and aged mice. However, rehabilitative-training induced reorganization is not seen in aged mice during the experimental period examined in the current study.

The existence of an additional ICMS-evoked forelimb area (RFA) located anterior to the primary motor area (CFA) in the rat motor cortex was first confirmed by Neafsey and Sievert (1982). Anatomically, the RFA is located mainly in the AGm, while the CFA is mostly located in the AGl and overlaps with the somatosensory representation in granular cortex (Hall and Lindholm, 1974). Horse radish peroxidase (HRP) labeling of corticospinal tracts shows that the CFA and RFA contain separate clusters of corticospinal neurons (Neafsey and Sievert, 1982). Anterograde and retrograde tracing studies show prominent connections between the RFA and the insular cortex (Rouiller et al., 1993) and from the AGm to AGl, visual, auditory, and limbic areas (Reep et al., 1987). This anatomical organization parallels the connections of the primate premotor and supplementary motor areas (Jurgens, 1984; Matelli et al., 1986) and the supplementary motor area of a larger rodent species, the porcupine (Lende and Woolsey, 1956).

Behavioral results following lesions of the RFA provide evidence that this area is functionally distinct from the CFA. Gharbawie et al. (2007) found that rats with lesions of the rostral motor cortex, inclusive of RFA, have only a slight, transient deficit in skilled reaching performance. In contrast, rats with lesions of the caudal motor cortex, inclusive of CFA, or the entire motor area show significant, chronic impairments in skilled reaching. Gharbawie et al. (2007) propose that these results indicate a lesser contribution of the RFA to skilled reaching performance in rats. Damage to the premotor cortex in humans results in deficits in learning and performing sequential motor movements (Dovern et al., 2011). Therefore, while RFA lesions in rodents may not have

as severe an impact on motor skill performance, they may result in a deficit in motor planning or reaching strategy, which may be evidenced by the early deficits in qualitative measures of reaching performance seen in rats with RFA lesions (Gharbawie et al., 2007).

In squirrel monkeys, lesions of the MI hand area result in enlargement of the hand representation within the ventral premotor cortex (PMv; Frost et al., 2003). Following lesions of both the MI and PMv, enlargement of the hand representation in the supplementary area (SMA) is observed (Eisner-Janowicz et al., 2008). In both areas, the enlargement is proportional to the amount of hand representation damaged by the lesion. Dancause et al. (2005) expanded on these results by determining that biotinylated dextran amine (BDA)-labeled neurons from the PMv send new axonal projections to SI following lesions of MI. This study supports the idea that the PMv seeks out new postsynaptic targets following degradation of its connections to MI and implicates SI as a potential substrate for adaptive plasticity following MI lesions. Evidence from Gharbawie et al. (2007) and these dissertation studies implicate the RFA as a potential site for adaptive plasticity in the rodent brain following CFA lesions. Rats with complete lesions of the forelimb areas of the motor cortex (inclusive of CFA and RFA) are more impaired than animals that only have damage to the CFA. Addition of a subsequent lesion damaging RFA worsened impairments in rats with prior CFA damage.

Overall, the anatomy, function and lesion-induced plasticity of the RFA show many similarities with the premotor and supplementary motor areas of the primate brain. These dissertation studies extend that this premotor or supplementary motor area

homology exists in the mouse brain and adds further support to the role of the RFA as a non-primary motor region capable of reorganizing following damage to primary motor regions.

6.7 Overall conclusion and future directions

These dissertation studies are the first to describe the organization of the motor cortex in the C57BL/6 mouse and to determine that motor skill learning is intact in aged mice while motor map plasticity is altered. These studies also established an endothelin-1 (ET-1) induced model of cortical ischemia in mice that results in long-lasting behavioral impairments that is suitable for furture studies utilizing transgenic strains of mice. Additionally, the final experiment of these dissertation studies suggests that aged animals are capable of remarkable behavioral improvements following focal ischemic lesions if they are provided with task-specific rehabilitative training. A possible contributing factor to the failure of older stroke survivors to regain skilled motor function is that larger lesions, as seen in aged mice, damage many of the surrounding tissues that would typically serve as substrates for motor map reorganization (Liu and Rouiller, 1999; Conner et al., 2005; Dancause, 2006). Although the effects of aging and lesion extent can not be dissociated in this study because the aged animals had significantly larger lesions than the young animals, future studies can use young and aged mice with similar lesion extents to study age-specific changes in behavioral improvement following stroke. Nevertheless, despite larger lesions and more persistent behavioral deficits following stroke, older adults can regain skilled motor function. Although we failed to see

reorganization of forelimb motor cortical representations, it is feasible that the aged motor cortex is capable of reorganization following ischemic injury, if given a longer duration of rehabilitative training, or that reorganization was not detected by the methods used in the study.

Although these dissertation studies used ICMS-evoked cortical responses as a measure of neuroplasticity, cortical remapping is hardly the only way in which the cortex changes following stroke. Future studies can use *in vivo* 2-photon microscopy to investigate the roll of dendritic spine turnover within perilesion cortex in behavioral improvement following stroke in the aged brain. Furthermore, repeated mapping of the motor cortex can now be conducted using newly-development light-based motor mapping of mice expressing channelrhodopsin-2 (ChR2) in layer V neurons of the motor cortex. This technology can be used to repeatedly map the forelimb representations within animals to determine the time-course of motor map changes due to motor learning or rehabilitative training following brain injury. Voltage-sensitive dye imaging can help to elucidate sensory or cognitive areas that are additionally activated following motor skill learning in the aged brain. Because these techniques are either more well-developed (i.e. voltage-sensitive dye imaging) or only available in mice (YFP and ChR2 transgenics), the model of motor learning and focal cortical ischemia developed in the preceding dissertation studies provides a useful model of brain aging suitable for use in imaging studies.

Appendix A

Table A1: Proportions of elbow, wrist and digit representations in the caudal forelimb area (CFA) by age and training condition (Chapter 3)

			% Elbow	% Wrist	% Digit	Summed Area (mm^2)
Young	Short duration	Controls	20.51 (6.69)	23.16 (4.22)	56.34 (7.47)	1.67 (0.14)
		Handlers	30.43 (6.02)	26.38 (2.75)	43.19 (5.05)	1.70 (0.20)
		Reachers	17.94 (6.74)	32.51 (5.14)	49.55 (7.59)	1.41 (0.17)
		Intensity Reachers	31.18 (13.27)	21.92 (10.82)	46.90 (14.28)	1.23 (0.10)
	Long duration	Controls	18.25 (4.33)	34.19 (5.44)	47.56 (6.74)	1.20 (0.12)
		Handlers	25.56 (0.08)	33.43 (6.30)	41.01 (5.27)	1.47 (0.12)
		Reachers	21.18 (0.07)	20.03 (4.15)	58.79 (5.92)	1.05 (0.07)
		Delay Reachers	31.34 (7.94)	29.30 (6.91)	39.36 (8.17)	1.48 (0.23)
Aged	Short duration	Controls	36.97 (5.68)	37.64 (3.03)	25.39 (6.34)	1.39 (0.21)
		Handlers	25.85 (6.05)	31.86 (5.88)	42.30 (8.75)	1.23 (0.15)
		Reachers	34.00 (7.38)	33.07 (7.57)	32.93 (6.70)	1.45 (0.20)
	Long duration	Controls	32.61 (7.11)	32.81 (9.44)	34.58 (10.15)	1.70 (0.28)
		Handlers	25.52 (5.77)	28.49 (5.55)	46.00 (7.39)	1.81 (0.36)
		Reachers	32.75 (3.58)	27.72 (5.28)	39.53 (5.85)	1.59 (0.18)

All data are means (S.E.M). The summed area includes the areal extents of elbow, wrist, and digit.

Table A2: Proportions of shoulder, elbow, wrist and digit representations in the caudal forelimb area (CFA) in long duration training conditions (Chapter 3)

		% Shoulder	% Elbow	% Wrist	% Digit	Summed Area (mm^2)
Young	Controls	19.53 (4.47)	15.21 (4.36)	26.94 (3.94)	38.32 (5.55)	1.52 (0.14)
	Handlers	21.51 (3.86)	20.23 (4.28)	25.96 (4.62)	32.30 (4.36)	1.89 (0.15)
	Reachers	27.35 (5.83)	14.89 (4.31)	14.22 (2.54)	43.54 (5.92)	1.5 (0.13)
	Delay Reachers	18.46 (6.00)	23.27 (4.84)	25.20 (4.84)	33.07 (7.49)	1.80 (0.23)
Aged	Controls	14.19 (4.81)	28.58 (6.81)	28.94 (9.16)	28.29 (7.46)	1.98 (0.28)
	Handlers	16.67 (4.18)	21.68 (5.43)	24.54 (5.39)	37.11 (4.64)	2.11 (0.32)
	Reachers	20.82 (5.57)	26.34 (4.12)	22.33 (4.93)	30.50 (3.63)	2.07 (0.30)

All data are means (S.E.M). The summed area includes the areal extents of shoulder, elbow, wrist, and digit. Shoulder representations were mapped in entirety only in long duration trained animals.

Table A3: Proportions of elbow, wrist and digit representations in the rostral forelimb area (RFA) by age and training condition (Chapter 3)

			% Elbow	% Wrist	% Digit	Summed Area (mm^2)
Young	Short duration	Controls	39.17 (17.46)	42.33 (14.16)	18.50 (15.56)	0.26 (0.07)
		Handlers	79.33 (16.14)	17.33 (12.93)	3.33 (3.33)	0.24 (0.06)
		Reachers	24.38 (14.19)	50.63 (17.18)	25.00 (16.37)	0.16 (0.03)
		Intensity Reachers	66.67 (33.33)	33.33 (33.33)	0.00 (0.00)	0.21 (0.08)
	Long duration	Controls	18.75 (13.15)	56.35 (17.52)	25.00 (16.37)	0.12 (0.02)
		Handlers	35.71 (10.76)	57.14 (11.82)	7.14 (7.14)	0.17 (0.02)
		Reachers	29.17 (17.18)	58.33 (14.43)	12.50 (12.50)	0.17 (0.07)
		Delay Reachers	45.24 (15.73)	54.76 (15.73)	0.00 (0.00)	0.14 (0.04)
Aged	Short duration	Controls	35.32 (15.21)	45.63 (15.16)	19.05 (16.36)	0.27 (0.07)
		Handlers	24.76 (14.16)	56.19 (17.33)	19.05 (14.29)	0.20 (0.05)
		Reachers	29.17 (23.94)	41.67 (25.00)	29.17 (23.94)	0.22 (0.07)
	Long duration	Controls	45.83 (20.83)	29.17 (17.18)	25.00 (25.00)	0.25 (0.09)
		Handlers	61.81 (17.89)	27.08 (12.99)	11.11 (7.03)	0.24 (0.07)
		Reachers	70.83 (15.02)	16.67 (16.67)	12.50 (12.50)	0.29 (0.11)

All data are means (S.E.M). The total area includes the areal extents of shoulder, elbow, wrist, and digit.

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